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Minicircular DNA as a potential gene therapy vector in cervical cancer

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"No que diz respeito ao desempenho, ao compromisso, ao esforço, à dedicação, não existe meio termo. Ou você faz uma coisa bem feita ou não faz."

Ayrton Senna

Para as pessoas mais importantes da minha vida e que eu mais amo,

Os Meus Pais

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Resumo Alargado

O Vírus do Papiloma Humano (HPV) infecta um elevado número de pessoas em todo o mundo, estando associado a mais de 99% do cancro cervical, e por isso, é considerado a segunda maior causa de morte em mulheres. O HPV é característico de inúmeras infeções e doenças associadas ao ser humano, dependendo do tipo de vírus envolvido. O HPV 16 é um vírus de alto risco pertencente ao género alfa-vírus do papiloma da espécie 9 e é o principal promotor da infeção do cancro cervical. O HPV 16 contém uma vasta diversidade genómica, sendo a proteína E6 responsável pela progressão do tumor devido à inativação da proteína supressora de tumor p53. A proteína E6 é considerada uma das proteínas oncogénicas do vírus, pois controla o ciclo celular nas células infetadas e estimula a proliferação celular. Assim, novas abordagens terapêuticas para o tratamento deste cancro têm sido exploradas, nomeadamente, a terapia genética que se baseia na inserção de material genético de interesse nas células, resultando na expressão das proteínas codificadas pelos respetivos genes.

O DNA plasmídico (pDNA) é um vetor não viral de baixa toxicidade e imunogenicidade, de fácil obtenção e manipulação, sendo por isso muito explorado em vacinas de DNA e em terapia génica. O plasmídeo contém na sua constituição sequências bacterianas essenciais à sua replicação no hospedeiro recombinante, como o gene de replicação, motivos CpG e marcadores seletivos de antibióticos, assim como a cassete de expressão eucariota constituída por um promotor, o(s) gene(s) de interesse e um terminador. Apesar de já terem sido descritos alguns casos de aplicação terapêutica de pDNA bem-sucedidos, atualmente, o uso de DNA minicircular (mcDNA) como vetor multigénico tem sido mais aprofundado uma vez que este apresenta alguns diferenças, que podem ser benéficas em relação ao pDNA. O mcDNA é constituído pela cassete de expressão eucariota, e não inclui as sequências bacterianas e motivos CpG, pois estes podem desencadear respostas imunológicas adversas e apresentar baixa biocompatibilidade e, ainda, não contém os marcadores seletivos de antibióticos que podem causar resistência na flora bacteriana humana. Assim, o mcDNA é um vetor de DNA biologicamente ativo, que além das suas dimensões serem inferiores ao pDNA, não manifesta efeitos citotóxicos inerentes por ser desprovido de sequências bacterianas, apesentando normalmente eficiências de transfeção e expressão de transgenes superiores aos níveis encontrados para o plasmídeo.

Portanto, o presente trabalho visa construir um vetor de mcDNA contendo o gene da p53 para restabelecer os níveis desta proteína supressora de tumor em células cancerígenas do colo do útero, de forma a induzir apoptose destas células. A produção de mcDNA começou pela clonagem da sequência codificante da p53 num plasmídeo parental (PP). A estirpe *Escherichia coli* (*E. coli*) Top 10 foi transformada por choque térmico com o vetor p53-PP, e após construção, o vetor foi sequenciado para obter a confirmação da inserção da sequência

codificante da p53 no vetor. Posteriormente, a estirpe *E. coli* ZYCY10P3S2T foi transformada por choque térmico com o vetor p53-PP. O uso desta estirpe de *E. coli* é de grande importância uma vez que esta permite a recombinação do PP, dando origem ao mcDNA e ao miniplasmídeo por indução com L-arabinose durante o crescimento celular. Além disso, no processo de recombinação, esta estirpe também digere o miniplasmídeo contaminante através da ação das endonucleases. De modo a obter um processo de recombinação eficiente, foi necessário otimizar este processo através de estudos no qual foram testados diferentes tempos de indução e diferentes concentrações do indutor L-arabinose. O melhor rácio entre mcDNA-p53/PP-p53 foi conseguido aplicando 0,01% de L-arabinose e 2 horas de indução.

Após produção, o mcDNA deve ser extraído e purificado para que possa ser avaliada a eficiência de transfeção e efeito celular deste vetor em estudos *in vitro*. Têm sido exploradas várias técnicas cromatográficas para a purificação de pDNA mas nem todas permitem obter resultados satisfatórios. Atualmente, a cromatografia de afinidade é a mais estudada para purificação de ácidos nucleicos porque é uma técnica que maximiza a recuperação e pureza do DNA, especialmente o pDNA na sua forma biologicamente ativa, a isoforma superenrolada (sc). Isto deve-se ao facto de a cromatografia de afinidade utilizar o reconhecimento molecular entre as moléculas alvo e os agentes de ligação específica tendo em conta a função biológica e estrutura química dos ligandos, como por exemplo, a utilização de alguns aminoácidos que mostram elevada especificidade e seletividade para reconhecer o pDNA. Por isso, neste trabalho foi estudada uma nova coluna cromatográfica de maneira a explorar o caráter de afinidade simultâneo de dois ligandos de aminoácidos, a arginina e lisina, imobilizados no mesmo grupo funcional, a triazina. Inicialmente foram testadas condições que favorecem maioritariamente interações iónicas ou hidrofóbicas nos passos de ligação/eluição, para se avaliar o perfil de interação entre a amostra e a matriz de modo a purificar o mcDNA-p53 sc. Os melhores resultados foram obtidos por duas estratégias diferentes, tendo-se revelado que uma favorecia a seletividade e a outra favorecia o rendimento de recuperação. Sendo assim, a estratégia de equilíbrio com menor concentração de sal parece favorecer a seletividade da matriz, obtendo uma amostra de sc mcDNA-p53 com menor teor de impurezas, embora a recuperação da amostra injetada não seja total. Por outro lado, o uso da estratégia de ligação efetiva da amostra à matriz, manipulando em simultâneo o pH, parece favorecer a recuperação do mcDNA-p53 na isoforma sc, apesar de conter algumas impurezas.

Nos estudos preliminares de transfeção *in vitro*, foram avaliadas a eficiência de transfeção dos vetores PP e mcDNA e a expressão da proteína supressora de tumor p53. Inicialmente, através de estudos de imunocitoquímica para visualização da expressão da proteína verde fluorescente, verificou-se que a transfeção das células cancerígenas do colo do útero (HeLa) ocorreu com ambos os vetores PP e mcDNA, tendo sido mais eficiente com o mcDNA. De seguida, foi confirmada a presença de transcritos da p53 através de RT-PCR e, por fim, confirmou-se pela técnica de Western Blot a expressão da p53 em células transfetadas.

Em suma, este trabalho permitiu a construção, biossíntese e recuperação de um vetor de DNA minicircular que codifica a proteína supressora de tumor p53, mostrando grande potencial para prosseguir com estudos de transfeção *in vitro* e *in vivo* no sentido de desenvolver futuramente uma estratégia para o tratamento do cancro do colo do útero.

Palavras-chave

Cancro cervical, cromatografia de afinidade, DNA minicircular, proteína supressora de tumor p53, terapia génica.

Abstract

The Human Papillomavirus (HPV) infects a large number of people worldwide and is associated with more than 99% of cervical cancer, being a second major cause of cancer death in women. HPV E6 oncoprotein is responsible for the progression of this tumor due to the inactivation of the p53 tumor suppressor. Thus, novel therapeutic approaches for the treatment of this cancer have been explored, for example gene therapy, which is based on the insertion of genetic material of interest into the cells, resulting in the expression of proteins encoded by the respective genes.

Plasmid DNA has been the non-viral vector mainly used in DNA therapeutics due to its simple manufacture, low toxicity and immunogenicity. Despite some successful cases, the possibility of using a DNA vector of reduced size, decreased toxicity by elimination of the bacterial cassette and able to favor the target gene expression by increased transfection efficiency, makes the minicircular DNA (mcDNA) a very promising gene therapy vector.

Therefore, the present work aims to construct a mcDNA vector encoding p53 to reestablish the levels of this tumor suppressor protein in cervical cancer cells and to induce apoptosis. The production of mcDNA started by cloning the p53-encoding sequence into a parental plasmid (PP). Then, *Escherichia coli* ZYCY10P3S2T strain was transformed by heat shock with the p53-PP vector. The use of this *E. coli* strain is of major importance since it allows the plasmid recombination to form mcDNA and miniplasmid species by induction with L-arabinose during cell growth. Moreover, after the recombination process, this strain is also able to digest the contaminant miniplasmid through endonucleases action. A highest mcDNA-p53/PP-p53 ratio was achieved by optimizing the recombination step to use 0.01% of L-arabinose and 2h of induction.

After the production, the mcDNA must be purified for further evaluation of transfection efficiency and cellular effect through *in vitro* studies. For that, a new chromatographic column was studied in order to explore the simultaneous affinity character of two ligands (arginine and lysine) immobilized in the same functional group (triazine), in which hydrophobic binding/elution conditions were explored and optimized in order to purify the sc mcDNA-p53. When it was used lower salt concentration in the equilibrium step, it seemed that the selectivity of the column was favored, obtaining a sample of sc mcDNA-p53 with lower content of impurities, although the total recovery of the injected sample has been sacrificed. On the other hand, using the strategy of effective binding of the sample to the column and manipulating the pH was seemed to favor the recovery of sc mcDNA-p53 despite containing some impurities.

In vitro transfection studies were performed to evaluate the transfection efficiency of PP and mcDNA vectors and the p53 expression, by using HeLA cells. Through immunocytochemistry it was observed that PP and mcDNA vectors were able to transfect

HeLa cells, being more efficient with mcDNA. By RT-PCR it was confirmed the presence of p53 transcripts and by western blot it was verified the correct expression of p53 tumor suppressor in transfected cells

Overall, this work allowed the construction, biosynthesis and recovery of a minicircular DNA vector encoding the p53 tumor supressor protein, showing great potential to proceed to *in vitro* and *in vivo* studies in order to develop a strategy for cervical cancer treatment in the future.

Keywords

Affinity chromatography, cervical cancer, gene therapy, minicircular DNA, p53 tumor suppressor protein.

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List of Abbreviations

µg	Microgram
µL	Microliter
µm	Micrometer
ºC	Celsius
APC	Antigens Presenting Cells
BCA	Bicinchoninic acid
DEPC	Diethypyrocarbonate
DNA	Deoxyribonucleic Acid
E. coli	*Escherichia coli*
E6AP	E6 Association Protein
EDTA	Ethylenediaminetetraacetic Acid
EU	Endotoxin Unit
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
g	Gram
gDNA	Genomic DNA
GFP	Green Fluorescent Protein
HCl	Chloride Acid
HPV	Human Papillomavirus
K_2HPO_4	Dipotassium Hydrogen Phosphate
KH_2PO_4	Potassium Dihydrogenphosphate
kbp	Kilo Base Pair
kDa	Kilo Daltons
L	Liter
LAL	Limulus Amebocyte Lysate
LB	Luria-Bertani
M	Molar
mA	Miliampere
mAU	Miliabsorbance units
mcDNA	Minicircular DNA
min	Minutes
mL	Milliliter
mM	Milimolar
mRNA	Messenger RNA
NaCl	Sodium Chloride
NaOH	Sodium Hidroxide

$(NH_4)_2SO_4$	Ammonium Sulfate
nm	Nanometer
oc	Open circular isoform
OD_{600nm}	Optical density at 600nm
ORF	Opening Reading Frames
p53	Tumor supressor protein
PBS	Phosphate buffered saline
PCR	Polimerase Chain Reaction
pDNA	Plasmid DNA
pKa	Acid dissociation constant
PP	Parental Plasmid
RNA	Ribonucleic Acid
rpm	Rotations per minute
sc	Supercoiled isoform
SDS	Sodium Dodecyl Sulfate
TAE	Tris, Acetic Acid, EDTA
TB	Terrific Broth
Tris	Tris(hydroxymethyl)aminomethane
UV	Ultraviolet
v/v	Volume/Volume
w/v	Weight/Volume

List of Scientific Communications

Oral Communication related with this thesis

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CHAPTER I

Chapter I - Introduction

1.1 Human Papillomavirus

Human Papillomavirus (HPV) is responsible for numerous infections and diseases associated with humans, depending on the type of virus involved [1, 2]. There are more than 200 papilloma viruses belonging to the *Papovariridae* family, in which they are divided into 5 genera depending on their differences in DNA sequences: alpha-virus, beta-virus, gamma-virus, mu-virus and nu-virus of papilloma (Figure A), being the first three the major groups. These groups can also be classified according to the location where they cause the infection: at the cutaneous level, which includes the beta-virus, gamma-virus, mu-virus and nu-virus of papilloma or at the level of mucosa where it stands out the alpha virus of papilloma [3, 4]. For the major group, the alpha virus of papilloma is further classified according to the risk of causing cervical cancer: low, moderate or high risk (Table 1). High-risk viruses are carcinogen agents, and are called human carcinogenic viruses [5].

Table 1. Human papillomavirus grouping, according to their risk to produce cervical cancer or precancerous lesions (adapted from [5]).

HPV group	HPV types
High risk	HPV-16, HPV-18, HPV-45, HPV-56
Intermediate risk	HPV-31, HPV-33, HPV-35, HPV-51, HPV-52, HPV-58
Low risk	HPV-6, HPV-11, HPV-42, HPV-43, HPV-44

HPV 16 is a high-risk virus belonging to the papillomavirus type 9 alpha virus and is the main promoter of cervical cancer infection [6]. About 61.35% of cases of cervical cancer are due to invasion by HPV 16 [4]. This cancer causes the death of about 0.25 million women per year, being considered the second most common cancer among women worldwide [2].

Human papillomaviruses are small size and feature approximately 8000 bp double stranded DNA complexed with histones [3, 5, 7]. HPV 16 contains a broad genomic diversity (Figure 1) [3]. It includes genes involved in replication, being the most common the E1 gene that encodes a DNA helicase for replication and amplification of the viral genome and the E2 gene whose role is to modify the normal function of cells for the benefit of the virus. It also has genes used in the packaging and structure of the virus capsule (L1 and L2 gene) and genes involved in the cell cycle control, such as the E4, E5, E6 and E7 genes. As for the structural

level, the protein viral particles have an icosahedral structure of 50 to 60 nm, which contains 360 molecules of L1 proteins organized in 72 capsomers. These structures are linked between the C-terminal of the L1 protein and the neighbor capsomer by disulfide bonds, thus forming the viral capsid. It may also contain some molecules of L2 proteins, but these are not fully exposed on the surface of virion [4, 7].

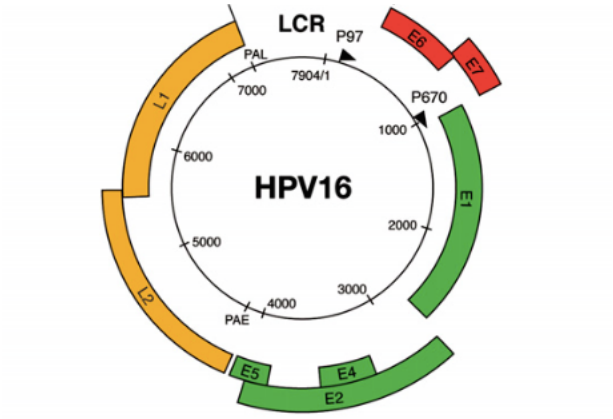


Figure 1. Human papillomavirus 16 (HPV 16) genome structure (adapted from [3]).

This virus penetrates the epithelium through microabrasions and infects the keratinocytes that are located in the basal layer of the epithelial cells. As a consequence, the virus will activate the cell cycle through infected and differentiated epithelial cells, in order to create a competent environment for replication and amplification of viral genome and coupling of the infectious particles [2].

The E6 and E7 proteins are considered the oncogenic proteins from the HPV 16 because they control the cell cycle in infected cells, stimulate cell proliferation and inhibit some aspects of innate immunity, specifically, they prevent interactions between epithelial and dendritic cells (antigens presenting cells) and block the production and responsiveness of interferons from infected cells [7, 8].

1.1.1 E6 Oncoprotein

The E6 oncoprotein is composed by 150 amino acids with 18 kDa approximately and it is localized in the nuclear matrix and non nuclear membrane fraction. As for the structural level, E6 consists by two zinc finger domains and in the base of each zinc finger are two motifs containing two cysteines (C-X-X-C, being the X any amino acid). In the second zinc finger

domain include an LXXLL motif, which is necessary to mediate interactions between the E6 protein and the other host proteins, specifically and commonly the cellular E3 ubiquitin-ligase known as E6-associated protein (E6AP). The interaction between E6 protein with E6AP results in loss of p53 activity from the infected eukaryotic cells [9], because the E6 oncoprotein leads to the degradation of p53 tumor suppressor protein mediated by ubiquitin binding, inhibiting apoptosis [10].

This mechanism has been reported for the high-risk HPV-16, while in the case of low-risk, E6 also binds p53 but with reduced efficiency, being unable of inducing p53 degradation. This tumor suppressor protein can sense stress or potential damage in cellular DNA and invoke a protective response by blocking the cell cycle progression or inducing apoptosis in the affected cell, which is extremely important to maintain the genome integrity and stability. When the p53 protein detects damage in the cellular DNA or oncogenic signals, it activates the WAF1/Cip1 gene, which is an inhibitor of cyclin-dependent kinases. High cellular levels of this complex WAF1/Cip1 inhibits cyclin-dependent kinases, which are important for progression through the cell cycle. Thus, the cell cycle stops in the G1 phase in order to repair the damage in the DNA and maintain the genome stability and cell integrity (Figure 2) [9].

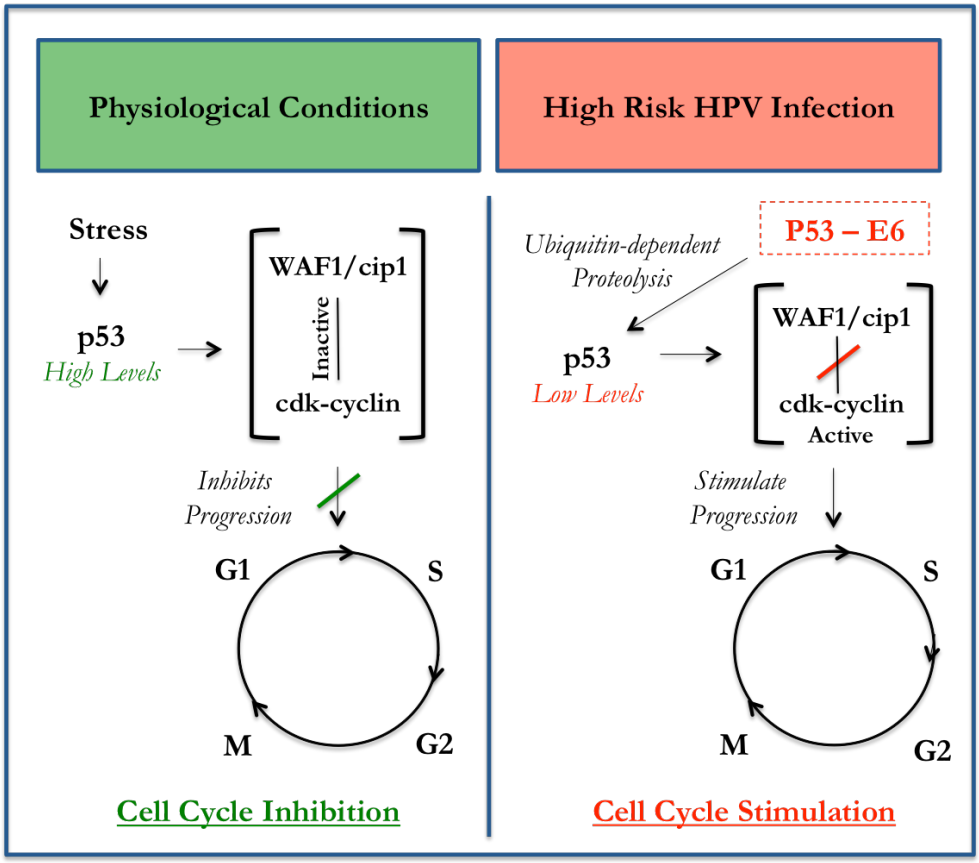


Figure 2. Effect of the interaction between E6 and p53 tumor supressor protein (adapted from [9]).

The E6 oncoprotein also leads to the activation of the catalytic protein subunit of human telomerase (hTERT). This endogenous enzyme takes care of maintaining the size of telomeres, and thus with each cell division the telomere does not diminish and cell can divide whenever it needs, making them permanently young. Therefore, with hTERT activation, the cells maintain the end of chromosomes, thus preventing cell senescence since the telomer size decrease leads to the rapid aging of the primary cells. [6].

Another problem caused by HPV infection is the inhibition of the immediate immune response. This occurs because the E6 oncoprotein inhibits epithelial-cell-dendritic-cell interactions and block the production of and responsiveness of infected cells to interferons.

Thus, this oncogenic protein, by inactivation or mutation of p53 function, triggers the uncontrolled proliferation of cells through continued replication of cells with damaged DNA promoting mutation, chromosomal instability and carcinogenesis of the host genome, along with the inhibition of apoptosis pathways and inhibition of the immediate immune response, leading to the start and progression of the tumor.

1.2 p53 – The Tumor Suppressor Protein

The inactivation of tumor suppressor p53 is one of the most common dysfunctions found in cancer and it is estimated that the p53 gene is mutated in nearly 50% of all known cancers [11]. It is a unique transcription factor, generally considered as the "guardian of the genome" due to its most essential feature that is the ability to inhibit cell proliferation [12]. The human p53, or TP53 is a nuclear phosphoprotein composed of 393 amino acids, 5 structural and functional domains: an N-terminal transactivation domain, a proline-rich regulatory domain, a sequence-specific DNA-binding domain, an oligomerization domain and a C-terminal domain involved in the regulation of DNA-binding (Figure 3) [11].

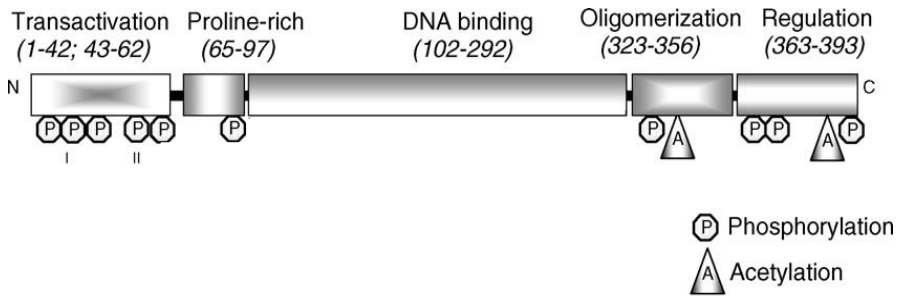


Figure 3. Schematic structure of p53 gene (adapted from [11]).

As regards the three-dimensional structure, the DNA-binding domain is made of a scaffold of beta-sheets that supports flexible loops and helixes. This characteristic allows to the direct contact with DNA. Thus, this domain is the most important not only due to the fact that it is the most mutated region in cancer pathologies but also because it is important to trigger apoptosis by annulling protein-DNA contacts or by disrupting protein folding [11].

The p53 signaling pathway is activated in response to a variety of factors such as stress, oncogenic signals or other damage, allowing it to coordinate transcription programs that ultimately contribute to tumor suppression. Thus, p53 allows to block cell growth and induce apoptosis, and it is therefore extremely necessary to maintain or restore their proper function in cells (Figure 4) [12].

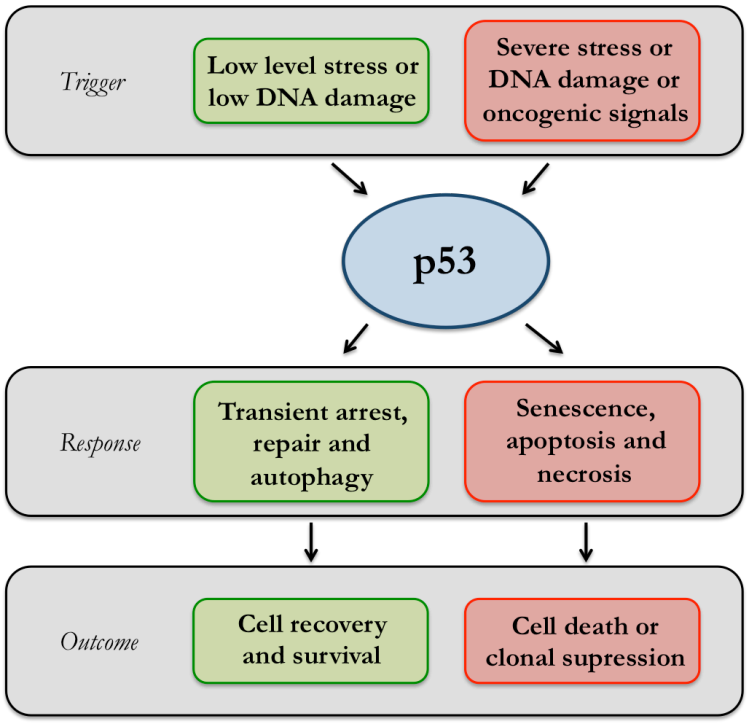


Figure 4. Outcome of p53 (adapted from [12]).

There are already several approaches with promising results, in which they aim to reactivate p53 through the use of drugs that may influence the mechanism of p53 control. As for example, a strategy already described showing good results in pre-clinical models is the use of molecules that inhibit the formation of p53 complex with its negative regulator MDM2. This occurs because MDM2 gene contains a p53-binding site that allows to bind tightly to p53 and inhibits its activity as a transcription factor. However, the majority of these approaches are

based on viral delivery systems, such as the first commercialized p53 cancer based therapy, which is used adenovirus for gene delivery [13].

To solve this problem, many therapies are used as new therapeutic approaches based on the delivery of DNA by using different type of vectors [14].

1.3 DNA-based Therapies

DNA-based therapies have been an interesting field under study due to their increasing impact over the last few years in various types of diseases. This therapeutic strategy became possible since decoding the genome. From this discovery, knowledge about genes and their expression products allowed the establishment of the relationship between genes and their respective expression products, mainly proteins, which can be associated to particular diseases [15].

For this approach, two therapeutic strategies have emerged with application of DNA: DNA vaccines and gene therapy. The first one intends to stimulate immune responses by delivering the genes encoding antigens for specific pathogens while gene therapy is based on the delivery of genetic material that encodes a desired gene, into the nucleus of malignant or mutated cell, resulting in its expression and subsequently, trigger its therapeutic effect [16, 17]. Both therapies have demonstrated potential in the evolution of clinical medicine being considered promising alternative therapies [16].

1.3.1 DNA vaccines

One of the greatest achievements of medicine was vaccination, since it allowed controlling the spread of numerous diseases. Significant developments have been observed in recent decades, but the efficacy of vaccines has to be controlled over time and their safety still represents a limitation [18]. The main goal of the vaccine is to prevent diseases in order to trigger an immune response against a pathogen. Presently, conventional vaccines are already marketed, which are based on dead pathogens, pathogenic subunits or attenuated viruses, but they are applied only to a small number of diseases. DNA vaccines have been developed to stimulate a strong and durable cellular and humoral immune responses through insertion of genes encoding antigens of specific pathogens. One of the major and important differences of DNA vaccines over conventional vaccines is their ability to transport highly conserved regions, allowing more effective fighting against pathogens that have suffered changes [19].

DNA vaccines have been demonstrating several advantages when compared to conventional vaccines, namely the increased safety, easier production and better specificity, bringing fewer obstacles since there is no risk of reversal of the virus activation that could trigger an infection. However, there are some disadvantages associated with this type of vaccine, such as low immunogenicity and low transfection efficiency. These problems appear because the delivery of naked DNA is not able to bypass biological barriers and it can be partially degraded or totally destroyed in the path to the cell nucleus (Table 2) [20, 21].

Table 2. Advantages and disadvantages of using DNA vaccines (adapted from [20, 21]).

Advantages	Disadvantages
Easy to store and transport	Low immunogenicity
Low cost production process	Risk of production of antibodies against the DNA administered
Possibility of prolonged exposition and action	Insertion of DNA with potential incidence of some cellular modifications
Low transfection efficiency or insufficient antigen expression	
Greater field of application	
No risk of return for virulent infections	
Induction of a immune response similar to that of vaccines with attenuated virus	
Immune response focus only on target antigen	

Currently, there are commercial preventive vaccines against HPV infection, with the principle of mimicking the infection caused by the virus so that the immune system memorizes specific functions to trigger in a future contact with the infectious agent and, thus, increase the immunity and protection against the virus [22]. However, these vaccines are not effective for already existing infections [23]. This has emphasized the need to develop not only preventive but also therapeutic vaccines in order to combat the proliferation of the virus and activate the humoral and cellular responses of the immune system through the insertion of genes encoding specific pathogens into multigenic vectors [24].

1.3.2 Gene Therapy

Gene therapy has been of increasing interest, due to the potential to treat a wide variety of acquired or inherited diseases, many of which are considered incurable, such as oncological, cardiovascular, infectious diseases and hereditary monogenic diseases (Figure 5). Nowadays, with the therapeutic successes, there was a growing development of this therapy, having now a more general applicability, with more than 1900 clinical trials in progress [25, 26].

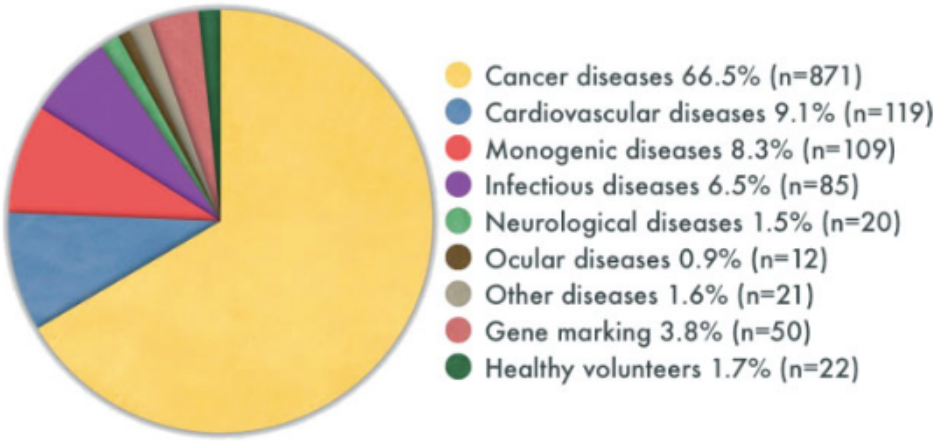


Figure 5. Graphical representation of the main areas of study for the application of gene therapy (adapted from [26]).

This therapy is based on the transfer of nucleic acids encoding the functional gene into the target cells, in order to replace or repair the mutated or deleted gene, thus regulating cellular procedures. So, gene therapy is only conceivable due to the ability of cells to capture new genetic information and express the encoded proteins. However, the correct identification of the gene suitable for the treatment of the disease and an efficient, precise and safe transport system is crucial [27].

As it is a therapy with genetic manipulation, regulatory control is of the highest importance because many ethical issues are raised, as well as safety concerns and possible adverse effects on the patient. Currently, legislation only allows gene therapy to be directed to somatic cells [25]. Therefore, gene therapy should result from a balance between safety and efficacy, and when these points are reached, this therapy could be applied for the treatment of various diseases [28].

1.3.3 Gene Delivery

For DNA-based therapies (i.e gene therapy and DNA vaccines) to become a real treatment option and to succeed, it is necessary an efficient delivery of genetic material to the target cells (Figure 6) [16]. Delivery systems represent an effective option to transport the DNA to the target cell, protecting it from degradation and facilitating the path to the cell nucleus [25]. Throughout this path, several obstacles may arise, such as the transport to the cell or the access into the cell nucleus, which limits the effectiveness of the therapeutic strategy [29].

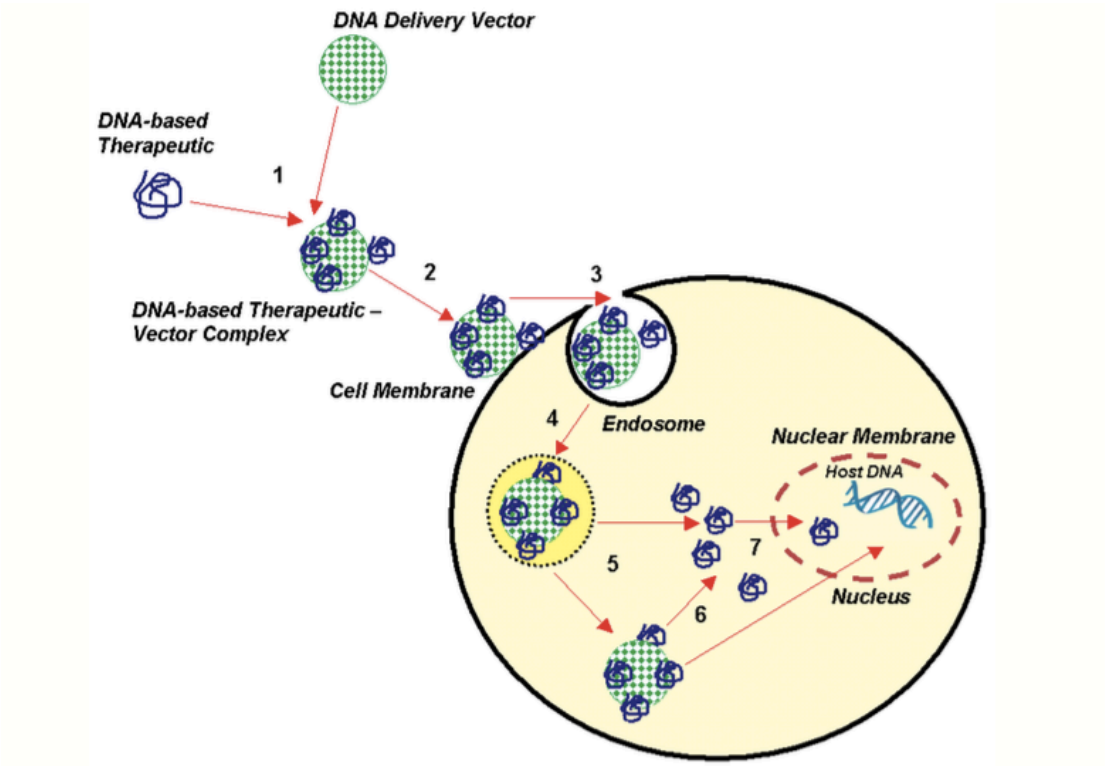


Figure 6. Schematic representation of the delivery of a DNA-based therapeutic using a DNA delivery vector (adapted from [16]).

In order to obtain the perfect delivery system and ensure maximum efficiency, the vector must meet the following prerequisites: to be innocuous and biocompatible, to avoid degradation by nucleases, detection by phagocytes and capture by the reticuloendothelial system; to present correct dimensions and ability to cross the plasma membrane and enter into the nucleus, where transcription will occur; and finally be safe and do not cause any harmful effect [25, 28].

One of the most essential factors to be taken into consideration when designing the vectors to be used is the targeting, because it must be ensured that these delivery systems are able to bypass biological barriers and move only to the target cells. If non-target cells are transfected during delivery process, some undesirable effects can occur in patients [30].

Delivery systems can be classified as viral and non-viral. Although both have advantages and disadvantages, non-viral vectors are a focus of increasing study because they are safer, present low immunogenicity and have a relatively simple manufacture. In studies based on gene therapy, the use of non-viral vectors has increased, but viral vectors such as adenovirus and retrovirus are still used (Figure 7) [26].

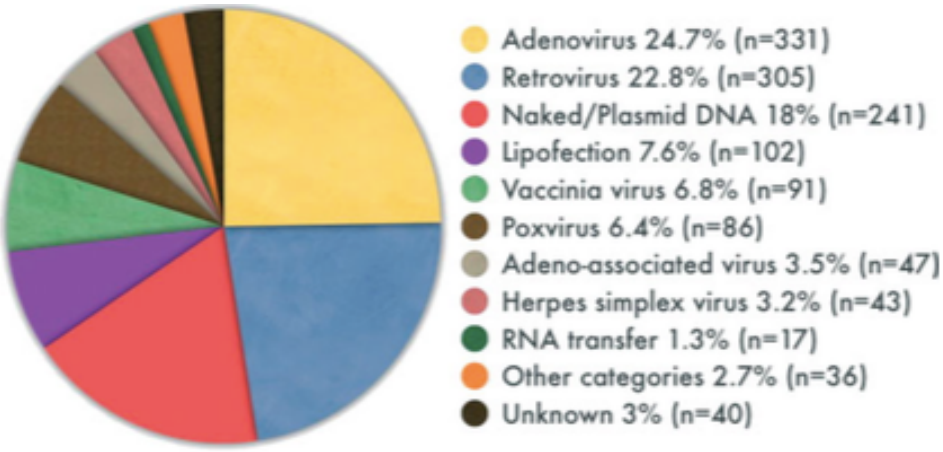


Figure 7. Graphical representation of gene therapy vectors used for delivery in clinical trials (adapted from [26]).

1.3.3.1 Viral Vectors

Viruses were the first delivery vectors used in DNA-based therapy because they present numerous properties that allow them to easily recognize and enter in eukaryotic cells, expressing their genes and consequently infecting the host cell [31]. This type of vectors has good applicability because they present high cellular evasion capacity, which facilitates the delivery of the genetic material. This ability can be exploited within the framework of gene therapy by replacing infectious and non-essential genes of the virus with therapeutic genes [30].

There are several viral vectors that can be used, such as adenovirus, adeno-associated virus, retrovirus and lentivirus, which present several advantages and disadvantages summarized in Table 3. Retroviruses were the first viral vectors to be described and, in this

case the safety of the vector is a major concern because they randomly integrate the genome of the host cell, which can lead to serious health complications. However, due to inherent advantages such as low pathogenicity, high infection efficiency and easy of handling, studies are still being done to improve its safety. Adenoviruses are double-stranded DNA viruses, being the most applied in the clinical context, since they present low pathogenicity and high capacity of protein expression. These vectors have the ability to infect several cell types and are easy to produce. However, they exhibit high immunogenicity and may trigger an immune response in the host. The adeno-associated vectors are small single-stranded DNA viruses and their replication is dependent on infection by a helper virus. Unlike adenoviruses, they have low immunogenicity and pathogenicity, low immune response by the host and high stability. However, they have the inconvenience of limiting the size of the therapeutic gene to be transported. Lentiviruses act on a wide variety of cells and present a reduced risk of mutagenesis, since they tend to integrate far from promoters, but yet present some pathogenic potential (Table 3) [32].

Table 3. Advantages and disadvantages of using viral vectors (adapted from [32]).

Vector	Advantages	Disadvantages
Retrovirus	Fairly prolonged expression High transfection efficiency Substantial clinical experience Low immunogenicity	Low transfection efficiency *in-vivo* Insert size limit of 8kb *ex-vivo* Transfects only proliferating cells Difficult manufacture and quality control Safety concerns (mutagenesis)
Adenovius	High transfection efficiency Transfects proliferating and non-proliferating cells Substantial clinical experience	Strong immune responses Insert size limit of 7.5kb Difficult quality control
Adeno-associated virus	Efficient transfection of wide variety of cell types *in-vivo* Prolonged expression Low immunogenicity	Difficult manufacture and quality control Insert size limit of 4.5 kb Limited clinical experience Safety concerns (mutagenesis)
Lentivirus	Transfects proliferating and non-proliferating cells Transfects hematopoietic stem cells	Very difficult manufacture and quality control Poor storage characteristics Insert size limit of 8 kb No clinical experience Safety concerns

Overall, despite the viral vectors are very efficient gene delivery vectors, these systems also present some disadvantages. These include safety concerns, such as the possible occurrence of mutagenesis and carcinogenesis, induction of immune responses, the low DNA amount that can be loaded and the high commercial cost. Considering these limitations, the use of non-viral DNA-based therapy has appeared as a convincing approach for the gene delivery [33].

1.3.3.2 Non-viral Vectors

In order to overcome the problems associated with viral delivery systems, non-viral vectors are being explored. They allow to improve some features, particularly, more safety, capacity for insertional mutagenesis, ability to transfer and deliver highest size genes. Other important characteristics in non-viral vectors are that they are less pathogenic and may have reduced toxicity in comparison with the existing viral vectors, and also include as advantage their easier production, with lower associated costs and higher stability (Table 4) [32].

Table 4. Advantages and disadvantages of using non-viral vectors (adapted from [32]).

Advantages	Disadvantages
Manufacture and quality control relatively simple Good storage characteristics Low immunogenicity Good safety profile Efficient transfection *ex-vivo* Delivery to any somatic cell Non-infectious No limit on size of plasmid	Short duration of expression Repeat therapy required as plasmid does not replicate with host cells Inefficient transfection *in vivo*

As already discussed, the entry of non-viral vectors into target cells may be difficult and limited, so they must overcome several biological obstacles in order to be able to exert their correct function. Thus, different methods have been developed, including the use of synthetic and natural materials, or even the use of physical forces, which facilitate the access of the genetic material into the cell nucleus. Chemical approaches use synthetic or natural compounds as carriers to deliver nucleic acids into the target cells. On the other hand, physical approaches employ a physical force that permeates the cell membrane and facilitates

intracellular gene transfer. Although a significant progress has been made for application of various delivery systems of non-viral vectors, these approaches also present some disadvantages and limitations (Table 5) [29].

Table 5. Advantages and disadvantages of delivery systems of non-viral vectors (adapted from [29, 34, 35]).

	Methods	Advantages	Disadvantages
Physical	Needle injection	Simplicity and safety	Low efficiency
	Gene gun	Good efficiency	Tissue damage in some applications
	Electroporation	High efficiency	Limited working range Need for surgical procedure for non-topical applications
	Hydrodynamic delivery	High efficiency, simplicity, effectiveness for liver gene delivery	Extremely effective in small animals Surgical procedure may be needed for localized gene delivery
	Ultrasound	Good potential for site-specific gene delivery	Low efficiency *in vivo*
Chemical	Cationic lipids	High efficiency *in vitro* Low to medium high for local and systemic gene delivery	Acute immune responses Limited activity *in vivo*
	Cationic polymers	Highly efficient *in vitro* Low to medium high for local and systemic gene delivery	Toxicity to cells Acute immune responses
	Lipid/polymer hybrids	Low to medium high for local and systemic gene delivery Low toxicity	Low activity *in vivo*
	Metallic nanoparticles	Magnetite and maghemite are relatively non-toxic and simple to prepare	Toxicity to cells Agglomeration Low sustainability High cost

In recent years, research in new non-viral systems has been intensified in order to develop efficient strategies of DNA-based therapy. In this case, the therapeutic gene delivery can be performed by using different classes of non-viral vectors, namely plasmids, cosmids, and artificial chromosomes [15]. The most used is the plasmid DNA (pDNA) and numerous studies have been developed by applying this modern technology [36].

1.4 Plasmid DNA

Plasmid DNA has stimulated a continuing interest mainly for application in gene therapy and DNA vaccines. In these therapeutic approaches, plasmid DNA is used as a vector to deliver a gene to the target cells and induce the production of relevant proteins and produce the expected therapeutic effect [36].

Plasmid DNA consists in a double-stranded circular DNA molecule, being mainly produced by using prokaryotic systems as hosts such as *Escherichia coli* (*E. coli*), isolated, purified and delivered through biotechnological processes [37]. Plasmids have the ability to naturally replicate, in an autonomous way into the bacterium and independently of the host cell cycle. As regards its structure, the pDNA is divided into hydrophilic backbone, composed by sugar and phosphate group, and hydrophobic interior of double helix, composed by planar bases stacked on each other. The phosphate groups of the pDNA molecule are negatively charged when the pH is higher than 4 [27].

In the two last decades, the plasmid DNA-based delivery vectors were the most popular non-viral systems used in clinical trials, gaining recognition for application in different diseases. This fact is probably due to the low toxicity, immunogenicity and high safety when compared with viral vectors. However, there are limitations in the use of this vector, such as low transgene expression levels through possible damages occurred in structural genes of eukaryotic expression cassette and the biological barriers mentioned before, which can reduce the transfection efficiency, resulting in the production of low levels of the target protein [38]. To increase expression levels, one of the strategies explored has been the isolation of the supercoiled (sc) isoform of plasmid DNA. This isoform is considered the most appropriate and bioactive form for therapeutic applications due to its functional, compact and undamaged structure. These factors allow better access to the nucleus of the target cell facilitating gene expression. Nevertheless, other isoforms can be generated from the sc pDNA isoform by either single strand nick, resulting the open circular (oc) isoform or double strand nick, which forms linear isoform. The existence of different pDNA isoforms also depends on other factors such as DNA sequence, stress or unfavorable environment conditions such as extreme pH or high temperatures [39].

In order to be acceptable the use of this sc pDNA isoform in therapeutic applications, it will be essential to conform all quality criteria recommended by regulatory agencies (such as FDA) in terms of the contaminant content present in the final sample (host constituents, namely endotoxins, genomic DNA, RNA and proteins) [40]. For that purpose, the biotechnological process involved in pDNA preparation is divided into two main steps: upstream process, which includes the production of pDNA by genetically modified organisms and downstream process that corresponds to the plasmid isolation and purification (Figure 8) [41]. Ultimately, it is important to make a balance between the development of strategies that allow a faster and most cost-effective production process without compromise the integrity of pDNA and a purification strategy that enables effective isolation of the plasmid in the sc isoform for a possible therapeutic application.

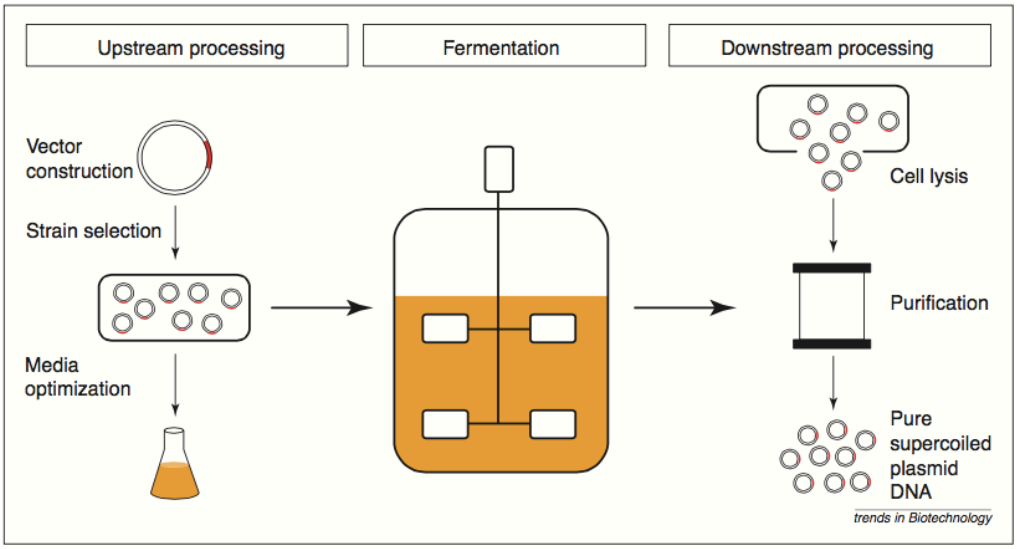


Figure 8. Plasmid DNA process development (adapted from [41]).

1.4.1 Upstream Process

As mentioned before, it is important to control the sc pDNA quality during the biotechnological process and fulfill the recommendations of regulatory agencies. For that, firstly it is needed a large-scale production, and then it is required the establishment of a suitable downstream process to guarantee a high purity degree. In parallel, it is also mandatory to select and apply analytical techniques sensitive enough to evaluate the vector's quality [24].

The design and engineering of pDNA is an essential step to ensure the successful pDNA manufacturing. The structure of plasmid DNA is divided into two major regions: the bacterial

amplification unit and the eukaryotic transcription unit. The first one contains the origin of replication (ori), which is crucial to occur an autonomous and efficient vector replication and in adequate quantities, an antibiotic resistance gene for growth selection of cells transformed with the vector, non-methylated CpG motifs that consists of DNA sequences composed by cytosines and guanines connected by phosphodiester bonds, which are useful to activate immune system and presenting antigens for DNA vaccine approaches. Finally, the second region of pDNA denominated by eukaryotic expression cassette contains the gene of interest for further expression in the eukaryotic cells, and the regulatory elements, such as promoters, enhancers and the polyadenylation termination sequence (PolyA), which is important for transcript stabilization and protection against RNA degradation [42, 43].

The next step consists in the choice of the bacterial strain and optimization of the fermentation conditions to enable the production of large quantities of sc pDNA under stable conditions. *Escherichia coli is* the ideal host for this type of use, because it has been described as very effective in producing pDNA since this bacterial strain have inherent advantages, namely the ability to reach a high cell density in a short time, reduced consumption of nutrients, economical and well genetically characterized in comparison to a wide variety of hosts. Another characteristic of this host is the capacity to allow the manipulation of fermentation conditions such as the composition of culture medium, temperature and pH, which is essential for application in different strategies of plasmid production [39].

Once the production step is completed, it is required the improvement of sc pDNA isolation and purification. It is fundamental to optimize all initial processes because the downstream process will be strongly influenced by previous steps [41].

1.4.2 Downstream Process

After upstream process, it is essential to release plasmids from the host cells and perform their separation from other cellular components, since the production is intracellular. Thus, downstream process is critical for impurity elimination, being an important step of pDNA recovery and isolation [41].

Cell lysis is the first step involved in this procedure, which can be performed by mechanical or chemical methods. Alkaline lysis is the most applied technique and is based on cellular membrane disruption by using a buffer containing NaOH to disrupt the hydrogen bonds, which also denatures proteins that are involved in maintaining cell membrane, followed by the release of all intracellular constituents (such as pDNA, RNA, genomic DNA (gDNA), proteins and endotoxins). On the other hand, this agent with high pH levels promotes the denaturation of gDNA, RNA and cell wall material. In particular, the sc pDNA isoform also relaxes in the presence of the NaOH, however, if the pH is lower than 12.5 prevents, during a short time, the

separation of complementary strands [44]. Subsequently, the formation of a precipitate with these contaminants occurs by addition of a neutralizing buffer, containing high concentration of potassium acetate, and finally the precipitate is removed by centrifugation. This process is considered a critical step in the recovery of sc pDNA and it is important to avoid plasmid loss and its conversion into other isoforms. Although the use of denaturation and precipitation processes during the alkaline lysis, the clarified sample resultant from this procedure still contains proteins, RNA, gDNA and endotoxins. To solve this problem, concentration and clarification steps should be performed to remove contaminants and increase the plasmid mass fraction. Therefore, in order to concentrate the plasmid by precipitation, it can be used an alcohol (such as isopropanol) and to clarify the sample by precipitation of high molecular weight impurities it can be used a salt (such as ammonium sulfate) [45].

Finally, it is crucial the application of a chromatographic technique to carry out the final pDNA purification. Chromatography has evolved in recent years and is considered one of the most efficient methods to obtain high quality and quantity of pDNA, respecting some requirements [44, 45].

1.4.3 pDNA Purification

The pDNA has to be purified in order to proceed with the therapeutic application, since a therapeutic product should present a high purity degree and quality, according to guidelines recommended by regulatory agencies (Table 6) [40].

Table 6. Specifications of pDNA quality, according to regulatory agencies (adapted from [40]).

Biomolecule	Guidelines
pDNA	>97% sc
Proteins	Not detectable
RNA	Not detectable
gDNA	<2 ng/ug plasmid
Endotoxins	<0.1 EU/ug plasmid

To separate the sc pDNA isoform from the other plasmid isoforms and host components, liquid chromatography is chosen as the central technique. The physical and chemical resemblances between host impurities and non-functional pDNA isoforms and sc pDNA represent

one of the major complications in its purification. Specifically, the similarities are the negative charge with RNA, gDNA and endotoxins, hydrophobicity with endotoxins and molecular mass with gDNA. Different chromatographic methods have been developed based on properties such as size, charge, hydrophobicity or affinity. Thus, this process have been optimized in order to ensure the structural and functional stability of the active sc pDNA and, at the same time, create a faster and more efficient separation, resulting in the maximum recovery yield [41, 46].

1.4.3.1 Size-exclusion chromatography (SEC)

Size-exclusion chromatography allows the pDNA purification from other molecules present in a lysate based on differences of their molecular size. SEC can be applied with high efficiency in separation of pDNA from RNA and endotoxins because RNA and endotoxins are smaller molecules that have greater ability to penetrate inside the pore space, retarding the movement through the column and their elution. However, separation of large molecules, such as gDNA and different isoforms of pDNA, is limited because these molecules travel around the particles of the column and are the first to be eluted, being difficult to isolate from sc pDNA [40, 46].

1.4.3.2 Anion-exchange chromatography (AEC)

This chromatographic method consists in the separation of biomolecules based on the attraction between opposite charges. Plasmid DNA is considered a polyanionic molecule that will interact with positively charged ligands immobilized in the stationary phase. Usually, the ligands used for pDNA purification by AEC are quaternary amines and the electrostatic interactions are predominant. For binding and elution of different molecules and impurities have been explored different salt concentration, and in this technique, it was already described that the binding step preferably occurs at low salt concentration. After binding, elution occurs by increasing salt concentration and it is dependent on the charge density, chain length and conformation of molecules. The separation of sc pDNA from other isoforms in an anion-exchange column can be possible with an optimized gradient due to the fact that sc is more compact and has a higher charge density than the oc or linear isoforms, being eluted later. However, it is necessary to take into account that the chemical and structural similarity between sc pDNA and the remaining biomolecules can limit their separation [44, 46].

1.4.3.3 Hydrophobic interaction chromatography (HIC)

Hydrophobic interaction chromatography is a method that explores and recognizes differences in hydrophobicity between nucleic acids and host impurities. The binding is achieved by column loading at a high salt concentration, because ionic strength is responsible to remove hydration water around hydrophobic groups, favoring the interaction between biomolecules and ligands. The elution of different biomolecules is accomplished by reducing the salt concentration, which weakens hydrophobic interactions and, thus, species are eluted by increasing order of hydrophobicity. In the case of pDNA purification, this method allows the evaluation of differences in hydrophobicity between double-strand and single-strand nucleic acids and host contaminants. Therefore, this chromatographic technique is efficient in separation of RNA and endotoxins, though, it presents low selectivity in relation to the different pDNA isoforms [46, 47].

1.4.3.4 Affinity chromatography (AC)

Affinity chromatography has become a popular method for biomolecules purification and it is the unique technique that uses specific binding agents to recognize and purify the target molecule based on their biological function or chemical structure [14, 37, 48]. AC has some advantages such as the possibility to reduce the number of steps, increasing yields and improving process economics. The ligands used in this process may be either natural or synthetic, although natural ligands present some limitations mainly in regard to its biological origin. In this way, to solve this problem, synthetic ligands have been developed, combining the selectivity of natural ligands with high capacity and durability of synthetic systems. This strategy separates biomolecules on the basis of reversible interactions between the target biomolecule and its specific ligand immobilized into the stationary phase. For that, the binding step starts by inject a sample containing the target biomolecule onto the stationary phase, with appropriate pH and ionic strength in the buffer. To obtain the target biomolecule, the elution steps can be performed specifically, with competitive agents, or non-specifically by changing buffer conditions, such as pH, ionic strength or polarity, depending on the characteristics of the biomolecules [44]. Different types of AC have been used for sc pDNA purification, such as immobilized metal-ion AC, triple-helix AC, polymyxin B AC, protein-DNA AC and amino acid-DNA AC. These AC types present advantages and limitations, although, amino acid-DNA has been extensively studied because of its ability to promote a set of interactions with nucleic acids (Table 7) [46].

Table 7. Affinity chromatography methods for purification of nucleic acids (adapted from [46]).

Affinity type	Principle	Specific binding	Advantages	Limitations
Immobilized metal-ion	Chelating ligands charged with divalent metal-ion specifically interact with aromatic nitrogen atoms through p-d orbital overlap	Single-stranded nucleic acids (particularly purine bases)	Efficient resolution of RNA from gDNA and pDNA; High endotoxin removal; Separation of denatured pDNA	pDNA in the flowthrough; Incomplete RNA capture in complex mixtures; Co-elution of all DNA forms
Triple-helix	Specific sequences present on DNA are recognized by an immobilized oligonucleotide, forming a triple-helix	Double-stranded DNA	Discrimination of different plasmids based on their sequence; sc pDNA isolation in one chromatographic step; Reduction of RNA, gDNA and endotoxin contamination levels; Possibility for scale-up	Loss of pDNA during washing step; Low yields; Slow kinetics of triple-helix formation; Long chromatographic run times
Polymyxin B	Immobilized polymyxin B specifically recognizes the lipid structure of endotoxins	Endotoxins	Elimination of endotoxin contamination from pDNA preparations	Non-specific interaction of ligands with pDNA; Poor yields; Toxicity of polymyxin B
Protein-DNA	A protein or protein complex immobilized on the matrix specifically recognizes a DNA motif	pDNA	Discrimination of different plasmids based on their sequence; pDNA isolation from clarified lysates; Elimination of proteins and RNA from preparation	Relatively low yields; Contamination with gDNA
Amino acid-DNA	Multiple interactions occur between immobilized amino acids and nucleic acids	sc pDNA	sc pDNA purification in a single chromatographic step; Efficient elimination of RNA, gDNA, proteins and endotoxins	Elution with high salt concentration and relatively low yields (for histidine)

1.5 mcDNA

As described before, pDNA present several characteristics that makes this a highly attractive biopharmaceutical product. However, there are some particularities that can represent a limitation in therapeutic applicability, such as bacterial sequences essential for replication in the host, which can lead to an inefficient therapeutic response and possible clinical complications [49]. Specifically, pDNA presents genes conferring resistance to an antibiotic that can spread to human bacterial flora through a horizontal gene transfer, placing biological safety at risk. Additionally, unnmethylated CpG motifs are a limitation in gene therapy because they are immunostimulating by promoting activation of Toll-Liker receptors, affecting the expression of therapeutic gene in the target cell, inducing inflammatory responses and cell death. On the other hand, after pDNA entry into the target cell, bacterial sequences may associate with histones creating a dense heterochromatin structure that may lead to transcription factors inaccessibility. Therefore, these sequences contribute to the reduction or silencing of gene expression, thus compromising the efficiency of pDNA in gene therapy. Finally, the bioavailability of this vector is compromised due to its high molecular size [42, 49].

In this context, minicircular DNA (mcDNA) appears as a promising alternative, since it comprises an improvement of pDNA, not containing these structural units fundamental for the production in the bacterial host, being considered safer and with capacity to promote high levels of gene expression [50]. The mcDNA was first isolated from *E. coli* by Cozzaelly, Kelly and Kornberg in 1968. In this innovating study, the minicircular DNA was described as a small molecule, which genetic information was limited to a small number of genes and whose biological function was unknown [51]. This biomolecule has been evolving and, currently, is classified as a non-viral vector that arises from higher molecular size plasmids by elimination of genes essential for bacterial maintenance and replication [52].

1.5.1 Production

The mcDNA presents a supercoiled structure and is characterized by the absence of bacterial sequences, thus, exhibiting high therapeutic potential [28]. This molecule results from the intracellular recombination of the parental plasmid (firstly produced in the recombinant bacterial host, such as *E. coli*), which will give rise to mcDNA and miniplasmid (mP) through a *recombinase* (Figure 9). The mP includes genes responsible for production in the bacterial host, and mcDNA contains the eukaryotic *cassette* responsible for the therapeutic gene expression [53].

For the PP recombination step, numerous procedures were tested in order to promote the formation of the desired biomolecules through the action of *recombinases* into specific sequences present in the PP. Initially, this process was carried out by using the *lambda integrase*, but it presented low yields in conversion of PP to mcDNA and mP with undesired levels of toxicity [54, 55]. Recently, another approach has been used, allowing more promising results, due to the fact that it is more efficient, simpler and with lower associated costs. The method consists in the use of *serine phiC31 recombinase* that acts at the attB and attP sites, promoting an unidirectional recombination. To occur conversion of PP into mcDNA and mP, it is used a recombination inducer, the L-arabinose, which promotes expression of serine *phiC31 recombinase* that leads the production of this biomolecules. Simultaneously, it occurs the activation of *SceI-I endonuclease* that promotes the cleavage and elimination of mP and PP residues, which are considered impurities [52].

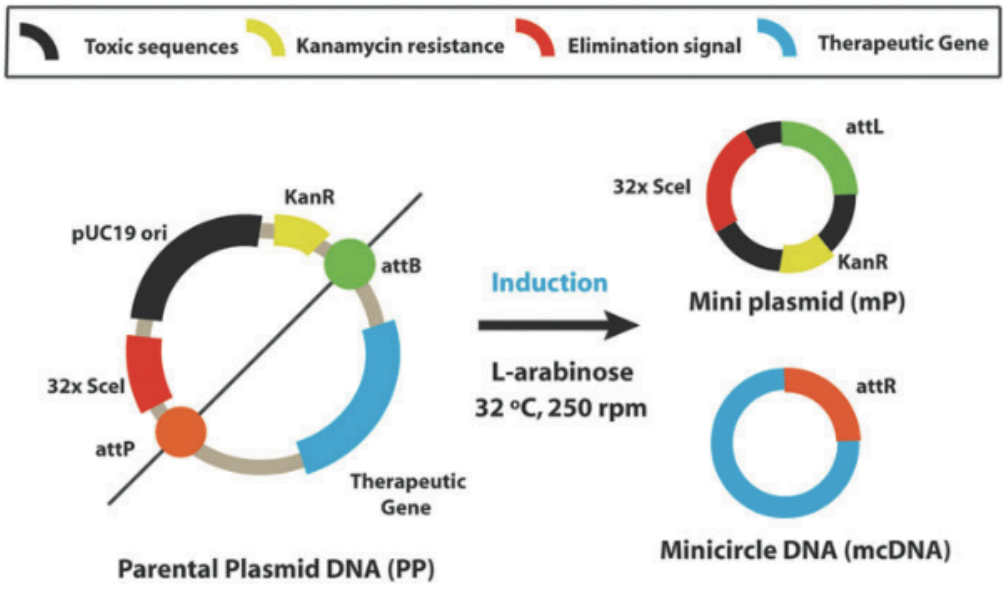


Figure 9. Schematic representation of mcDNA-producing technology from PP (adapted from [53]).

The mcDNA production can be influenced by different parameters such as temperature, induction time and the inducer concentration. The optimization of these factors is crucial because they can influence the levels of mcDNA and impurity production. Previous studies have described that the induction time and the inducer concentration depend on the implemented recombination process. For instance, it was shown that a production at 42ºC followed by an L-arabinose induction process at 32ºC increases the yield of mcDNA production [53].

However, from the recombination process, amounts of mP and PP can still remain, suggesting that recombination was not complete. Therefore, a purification procedure is required to isolate mcDNA and obtain a vector with potential application in therapy [56].

1.5.2 Purification

The implementation of a strategy that allows the purification of mcDNA and subsequent application in therapeutic approaches has been a huge challenge for researchers, due to the fact that these developments face difficulties imposed by the sample complexity and similarity between the biomolecules present in the original sample, which contains the mcDNA and other impurities such as PP and mP. Thus, some methodologies have been tested [49, 56].

Restriction enzymes have already been used to eliminate PP and mP residues, but the isolation of mcDNA has not been well achieved and the use of restriction enzymes may compromise the application on industrial and therapeutic scale [57]. Another method for mcDNA purification is the application of density gradients by using ethidium bromide and cesium chloride that are not conceivable in clinical context due to their toxicity [54].

On the other hand, a mcDNA purification strategy was implemented, based on affinity chromatography through interactions between proteins and DNA, in which occurs interaction between the lactose repressor and the respective operon. This technique requires the incorporation of the modified lactose operon into the mcDNA to be recognized by the immobilized protein into the matrix. The mcDNA elution occurs by the addition of a competitive agent that will lead to the breakdown of the established interactions. This strategy has the disadvantage of involving a previous step of incorporating lactose operon into the mcDNA, so that it can establish interaction with the chromatographic matrix [58]. Therefore, it will be essential the development of an efficient purification technology that allows the mcDNA isolation, complying the conditions imposed by the regulatory agencies.

CHAPTER II

Chapter II – Aim of the thesis

The present work proposes the construction of a mcDNA vector encoding the p53 gene, to reestablish the levels of this tumor suppressor protein in cervical cancer cells. Thus, after the construction step by molecular biology methodologies, it is necessary to optimize the recombination process of the parental plasmid into mcDNA through the addition of different concentrations of L-arabinose inducer at different induction times, in order to obtain a less complex and impure sample facilitating the mcDNA purification process. Given that the production of mcDNA is intracellular, it is necessary to carry out alkaline lysis followed by pre-purification steps. Then, the next step is focused on the application of an efficient downstream process to isolate and purify the mcDNA. In this context, affinity chromatography will be exploited by using a new chromatographic column comprising two ligands (arginine and lysine amino acids) immobilized in the same functional group (triazine). The binding/elution conditions such as pH, concentration or salt type must be manipulated in order to isolate the mcDNA sc isoform during the chromatographic steps. This purified biomolecule may subsequently be evaluated regarding its purity. The purified sample will then be applied in *in vitro* transfection studies of cervical cancer cells to evaluate the expression of the p53 tumor suppressor encoded in mcDNA.

CHAPTER III

Chapter III – Materials and Methods

3.1 Materials

For the vector construction through enzymatic digestion and cloning, enzymes XbaI, BamHI and T4 DNA ligase were used, respectively, and obtained from Takara Bio USA - Clontech Laboratories, Inc.. For the PCR methodology, Taq Polimerase and $MgCl_2$ were obtained from NZYTech, Lda.- Genes and Enzymes, Lisbon, Portugal. To the vector dephosphorylation, the enzyme CIP was purchased from New England BioLabs, Ipswich, MA. During the production, a bacterium *E. coli* DH5α transformed with the pcDNA3-FLAG-p53 and a bacterium *E. coli* ZYCY10P3S2T transformed with the vector pMC.CMV-MCS-EF1-GFP-SV40 polyA were used and obtained from the company System Biosciences. Reagents used for the bacterial cultures (tryptone and yeast extract) were purchased from Bioakar Diagnostics and the LB medium purchased from Panreac. For fragment and vector purification it was used NucleoSpin Gel and PCR Clean-up kit obtained from Macherey-Nagel & Co., Germany. For alkaline lysis and pDNA purification, the NZYTech Plasmid Maxi kit was used. In electrophoresis technique, it was used Greensafe Premium obtained from NZYTech, Lda.- Genes and Enzymes, Lisbon, Portugal. The buffers used for chromatographic assays were pre-filtered with membranes whose pore size was 0.2 μm (Schleicher Schuell, Dassel, Germany) and degassed by ultrasonic system. For concentration of the samples obtained in the chromatographic experiments, Vivaspin® concentrators were used (Vivaproducts, Littleton, MA, USA). The solutions used were prepared with ultra-pure deionized water, purified with the Millipore Milli-Q system (Billerica, MA, USA).

3.1.1 Plasmid DNA

The 6.59 kbp pcDNA3-FLAG-p53 plasmid was purchased from Addgene (Cambridge, MA, USA). This vector encodes for the human p53 protein conjugated with a FLAG tag and contains the ampicillin resistance gene and the SV40 virus mammalian expression promoter.

Figure 10. Plasmid pcDNA3-FLAG-p53 vector Map (adapted from Addgene).

3.1.2 Parental Plasmid

The 7.06 kbp pMC.CMV-MCS-EF1-GFP-SV40Poly A was purchased from Addgene (Cambridge, MA, USA). The vector contains the kanamycin resistance gene, the CMV7 promoter and the recombination sites attB and attP.

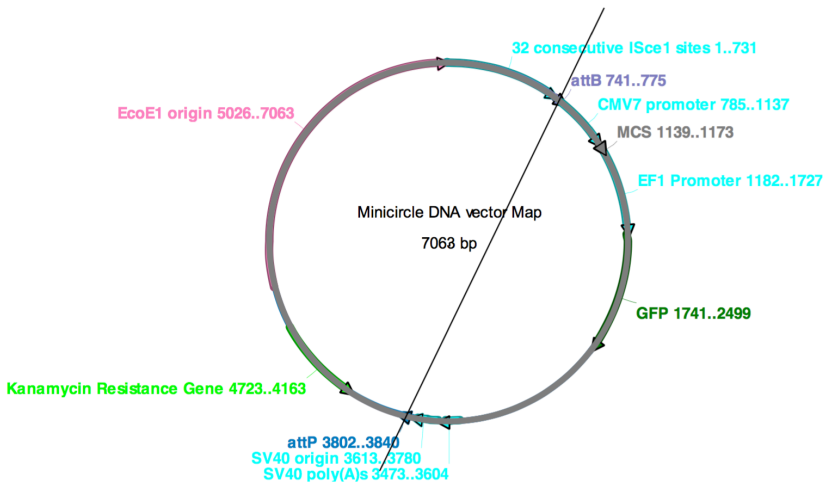


Figure 11. Parental Plasmid (PP) vector Map (adapted from Addgene).

3.2 Methods

3.2.1 Bacterial growth conditions

The pcDNA3-FLAG-p53 plasmid used for the p53 fragment amplification was produced by first inoculating the strain into LB-agar plates (30 µg/mL, ampicilin). After growth on solid medium, the fermentation started from a pre-fermentation (OD_{600nm} = 2.6) so that the initial OD_{600nm} of the fermentation was 0.2. The bacterial growth was carried out by a cell culture of *E. coli* DH5*a* in an orbital (Agitorb 200 IC, Aralab) at 37 °C, with agitation of 250 rpm in a 1 L erlenmeyer with 250 mL of complex Terrific Broth medium (20 g/L of tryptone, 24 g/L of yeast extract, 4 mL/L of glycerol, 0.017 M KH_2PO_4, 0.072 M K_2HPO_4) supplemented with 30 µg/mL of ampicillin. Growth was suspended at late log phase ($OD_{600nm} \approx 8$). At this stage the pcDNA3-FLAG-p53 was obtained, and the bacterial samples were collected by centrifugation (10 min, 4 °C, 450 g) and stored at -20 ºC.

The pMC.CMV-MCS-EF1-GFP-SV40Poly A plasmid used as the base vector was produced by the same way than pDNA3-FLAG-p53 vector, but using a cell culture of *E. coli* ZYCY10P3S2T supplemented with 50 µg/mL of kanamycin at 42ºC. In this case, the growth was suspended at late log phase ($OD_{600nm} \approx 5$).

3.2.2 PCR Amplification

To obtain the p53 fragment, the Taq Polimerase enzyme (NZYTaq2x Green MasierMix separate $MgCl_2$) and specific primers (StabVida) at 100 pmol (Table 1) were used. In a PCR tube, 6.25 µL of enzyme Taq Polimerase, 0.6 µL of primer forward (diluted 1:20), 0.6 µL of primer reverse (diluted 1:20), 2 µL of $MgCl_2$, 1 µL of pDNA (diluted 1:100) was included in a tube and water was added until a final volume of 12.5 µL. After mixing, incubation and amplification was carried out at 95 ºC for 5 min, (95 ºC for 40 sec, 60 ºC for 30 sec and 72 ºC for 1 min) x 30 cycles and finally at 72 ºC for 5 min in a thermocycler. The results were analyzed by 0.8% agarose gel electrophoresis after purification with a PCR Purification Kit.

Table 8. Primers for p53 fragment amplification.

Primer	
Forward	5'- AAT CTA GAA TGG AGG AGC CGC AGT CAG ATC -3'
Reverse	5'- ATG GAT CCT CAG TCT GAG TCA GGC CCT TC -3'

3.2.3 Cloning Step

For the enzymatic digestions of p53 fragment and Parental Plasmid, XbaI and BamHI restriction enzymes were used. In a PCR tube, 1 µL of enzyme, 2 µL of 10x buffer, 1 µg of pDNA or p53 fragment amplified was mixed and water was added until a final volume of 20 µL. After the preparation, incubation was carried out at 37 ºC for 2 hours in a thermocycler. The results were analyzed by 0.8% agarose gel electrophoresis after purification with a PCR Purification Kit.

Before the cloning step, it was performed the dephosphorylation of the digested PP vector, by using the CIP enzyme. In a PCR tube, 1 µL of enzyme, 2 µL of 10x buffer (NEB3), 1 µg of PP, already digested by XbaI and BamHI, were mixed and water was added until reach a final volume of 20 µL. Then the mixture was incubated at 37ºC for 1 hour in a thermocycler. The results were analyzed by 0.8% agarose gel electrophoresis after purification with a PCR Purification Kit.

The cloning mixture was based in a modified cloning protocol by Promega, in which it was fixed the concentration of the vector (digested and dephosphorylated PP) to 100 ng and was variated the concentration of insert (p53 digested fragment) through different ratios calculated by this equation:

$$\text{ng of insert} = \frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \frac{\text{insert}}{\text{vector}}$$

Thus, the ratios 1/3, 3/1, 1/1 of insert/vector were tested. The reaccional mixture was prepared in a sterile eppendorf tube, where 2 µL of enzyme ligase, 2 µL of 10x buffer, 100 ng of vector and ***x*** ng of insert, calculated by the previously equation, were included and water was added up until a final volume of 20 µL. After the preparation, incubation was carried out at 4 ºC for 18 h (overnight). To finalize the cloning process, the next step was the transformation of *E. coli*, and for that, the mixture was incubated to room temperature for 25 minutes.

3.2.4 Preparation and Transformation of *E. coli* competent cells

The Ionue method for the preparation of ultra-competent *E. coli* cells was applied, which is based on a slower growth of the bacterial culture by inducing thermal stress by decreasing the growth temperature [59]. An isolated bacterial colony was selected from a plaque that was previously inoculated for 16 hours at 37 ºC, into 25 mL of SOB medium (2 % w/v tryptone, 0.5 % w/v yeast extract, 8.56 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$ and 10 mM $MgSO_4$) in a 250 mL erlenmeyer. It was incubated for 6 hours at 37ºC with vigorous shaking (250 rpm), and at the end, this culture was used to inoculate another 250 mL erlenmeyer with 62.5 mL of SOB medium and 500 uL of the initial culture. This bacterial culture was incubated overnight at 20 ºC, 250 rpm. The OD_{600} was measured until reaching 0.55, and, then, the bacterial growth was stopped and the erlenmeyer was transferred to a cold bath for 10 minutes. To become competent, cells were collected by centrifugation at 2500 g for 10 minutes at 4 ºC, followed by carefully resuspension in 20 mL ice cold ITB (55 mM $MnCl_2 \cdot 4H_2O$, 15 mM $CaCl_2 \cdot 2H_2O$, 250 mM KCl, 10 mM PIPES – 0.5 M at pH=6.7), and finally a centrifugation at 2500 g for 10 min at 4 ºC. To complete the procedure, the cells were carefully resuspended in 5 mL of ice cold ITB and 375 ul of DMSO was added, and it was gently blending the bacterial suspension, being lastly placed on ice for 10 minutes. Finally, 100 µl of the cell suspension was transferred into cold sterile tubes and the competent cells were frozen rapidly by submerging the hermetically sealed tubes in liquid nitrogen and then stored at -80 ºC.

E. coli competent cells were transformed by heat shock with the p53-PP vector. The process starts by adding the PP-p53 to 100 µL of *E. coli* competent cells Top 10/ ZYCY10P3S2T and incubating on ice for 30 minutes. Next, the mixture was incubated at 42 ºC for 45 seconds and, subsequently, incubated at ice for 2 minutes. This mixture was added to a 15 mL sterile falcon tube with 400 µL of SOB medium in a orbital (Agitorb 200 IC, Aralab) at 37 ºC, 1 hour, 250 rpm. Finally, 250 µL of this bacterial culture was inoculated into LB-agar plates (50 µg/mL, kanamycin) and finally it was evaluated the bacterial colonies by PCR.

3.2.5 Optimization of mcDNA-p53 synthesis

For the production of mcDNA, an induction mixture was added to bacterial cultures, when the fermentation reached an optical density (OD_{600nm}) of approximately 5, consisting in a final volume of 250 mL of LB medium, 10 mL of 1 M NaOH with the pH adjusted to 7. L-arabinose (20% w/v) was used up to a final concentration of 0.001% or 0.01% or 0.1%, to find the best conditions. The recombination process was run for different hours (2, 3 and 4 hours) in a cooled orbital with constant temperature at 32 ºC and stirring at 250 rpm. The end of this

procedure, consist of the mcDNA obtention by centrifugation of the bacterial samples (10 min, 4 ºC, 450 g) and the cells stored at -20 ºC.

3.2.6 mcDNA-p53 recovery

For mcDNA and PP extraction, NZYTech Plasmid Maxi kit was used, including some modifications to the protocol provided by the manufacturer. Initially, the bacterial pellet was resuspended in 20 mL of P1 buffer (50 mM of Tris-HCl at pH=8.0, 10 mM of EDTA hydratated and 100 µg/mL RNase A). Thereafter, 20 mL of P2 buffer (200 mM of NaOH, 1 % of SDS (w/v)) was added in order to promote the lysis of cells. The tubes were gently homogeneized and incubated at room temperature for 5 minutes. The lysis step was stopped with the addition of 20 mL of P3 buffer (3.0 M of Potassium Acetate at pH=5.0). Next, incubation on ice was promoted for 20 min, followed by two centrifugations, 30 and 15 minutes (20000 g and 4 ºC) that resulted in the elimination of some precipitated contaminants, such as cell debris, gDNA and proteins. The supernatant was added to a anion exchange column, previously equilibrated with QBT Buffer (750 mM NaCl, 50 mM MOPS, 15 % isopropanol (v/v), 0.15 % Triton X-100 (v/v) at pH=7.0), and the proteins and low molecular weight molecules were initially removed by the addition of a buffer with low pH and salt content (0.90 M of NaCl). Next, elution of the mcDNA was promoted by the addition of a buffer with higher pH and higher salt concentration (1.75 M of NaCl). The species obtained in this step were precipitated by the addition of 0.7 volumes of isopropanol, followed by incubation on ice for 20 minutes. The obtained sample was subjected to centrifugation (16000 g, 4 ºC and 30 min) and the obtained pellet was resuspended in 1 mL of Tris-EDTA buffer (10 mM at pH=8.0). In this protocol, washing and elution buffers applied to the purification columns had a concentration of 1M and 1.25M, respectively. These concentrations are normally applied in conventional plasmids such as pDNA-FLAG-p53. However, to mcDNA recovery, it was necessary some changes at these buffer concentrations because under these concentrations, it was observed loss of mcDNA in the washing and elution steps. Therefore, after some optimizations by the group it was verified that the best concentrations in the washing and elution buffers were 0.9M and 1.75M, respectively. These concentrations allowed lower loss of mcDNA sample and, at the same time, to obtain a final sample with the minimum amount of contaminants. These modifications to the initial protocol of NZYTech kit suggest that mcDNA promotes stronger interactions with the ion exchange kit columns than pDNA probably because this biomolecule is smaller, compact and has more density of charges per surface area than pDNA. Thus, due to the intensity of interactions established by the mcDNA, higher ionic strength was necessary in the elution step to disrupt this binding. This procedure resulted in samples of mcDNA and PP that were later used in chromatographic assays for the isolation of the supercoiled isoform of mcDNA.

3.2.7 mcDNA-p53 purification - Affinity chromatography

The chromatographic studies were carried out in the AKTA Purifier system (GE Healthcare Biosciences, Uppsala, Sweden) with UNICORN software 5.11. This system consists of a compact separation unit and a computer. A new chromatographic column was studied in order to explore the simultaneous affinity character of two ligands (arginine and lysine) immobilized in the same functional group (triazine). For the mcDNA purification, the column was equilibrated with and without the presence of salt at a flow rate of 1 mL/min. When the equilibrium conditions were reached, the sample obtained from the lysis was injected using a 200 µL loop. After injection of the sample under these conditions the binding of the sample to the column was studied and, subsequently, different gradients were applied in order to promote the elution of the retained biomolecules. The fractions corresponding to each peak were recovered, concentrated by centrifugation, dessalted and then analyzed by agarose gel. All chromatographic assays were performed at room temperature and the absorbance was continuously monitored at 260 nm. At the end of the chromatographic assays, the column was regenerated by using a flow rate of 0.5 mL/min with 0.1 M NaOH in 30 % isopropanol (v/v) and, finally, the column and the AKTA Purifier system were cleaned with Mili-Q water.

3.2.8 Chromatographic column synthesis

The chromatographic matrix preparation and modification with specific ligands was performed by using the protocol already described for similar matrices [60]. Briefly, epoxy-activated agarose was produced by reacting SepharoseTM CL–6B with 10 M NaOH (0.04 g/mL moist agarose) for 30 min at 34 ºC, followed by addition of epichlorohydrin (0.072 g/mL moist agarose) for 3 h at 34 ºC. To continue with the column preparation, it was necessary to determine the amount of epoxy groups through incubation of 1 g of epoxy-activated agarose with 3 mL of an aqueous solution of 1.3 M sodium thiosulfate for 20 min at room temperature with agitation, and, then epoxy groups were quantified by titration with 0.1 M HCl. Before functionalizing the agarose with the triazine, epoxy-activated agarose was washed with Mili-Q H_2O, resuspended in 5 M NH_4OH (1.5 g/mL moist resin) and incubated overnight in the orbital shaker at 40 ºC. Aminated agarose was then washed with Mili-Q H_2O and resuspended in a cold solution of 50 % (v/v) acetone/Mili Q H_2O with 1 molar equivalent of $NaHCO_3$ relative to epoxy (1 g/mL agarose). The functionalization step was started by dissolving cyanuric chloride, which is a chlorinated derivative of 1,3,5-triazine in acetone (8.6 g/mL cyanuric chloride) with 5 equivalent molar excess relative to epoxy content. After that, it was added to the aminated resin, followed by incubation for 1h in ice with agitation. Once again, after incubation step, the

triazine-functionalized resin was washed by 3 steps: first 2 times the resin volume with acetone, second 3 times the resin volume with 50% (v/v) acetone/Mili-Q H_2O and, finally, 5 times the resin volume only with H_2O. The cyanuric chloride-functionalized agarose was then placed in flasks and the amino acid binding was started by adding arginine with 2 molar equivalent relative to epoxy (0.5 mL/well), an upper cover was placed and the flask was incubated for 24 h at 30 ºC with agitation at 100 rpm. Afterwards, the ligand was washed 5 times with 0.75 mL of the solvent in which the amino acid was dissolved and, next, 5 times with 0.75 mL of Mili-Q H_2O and the solvent was left to drain by gravity. The filter plate was bottom-capped and the second amino acid, lysine, was then added to each row of the filter plate with 5 molar equivalent relative to epoxy (0.5 mL/well) and incubated for 48 h at 80 ºC in the orbital shaker at 100 rpm (Figure 23).

In the end, to finish the matrix preparation, the functionalized resin was washed 5 times with 0.75 mL of the solvent in which each amino acid was solubilized, followed by 1 time with 0.75 mL of 0.1 M HCl, 1 time with 0.75 mL of Mili-Q H_2O, 1 time with 0.75 mL of 0.1 M NaOH in 30 % isopropanol (v/v), and, finally, 5 times with 0.75 mL of Mili-Q H_2O.

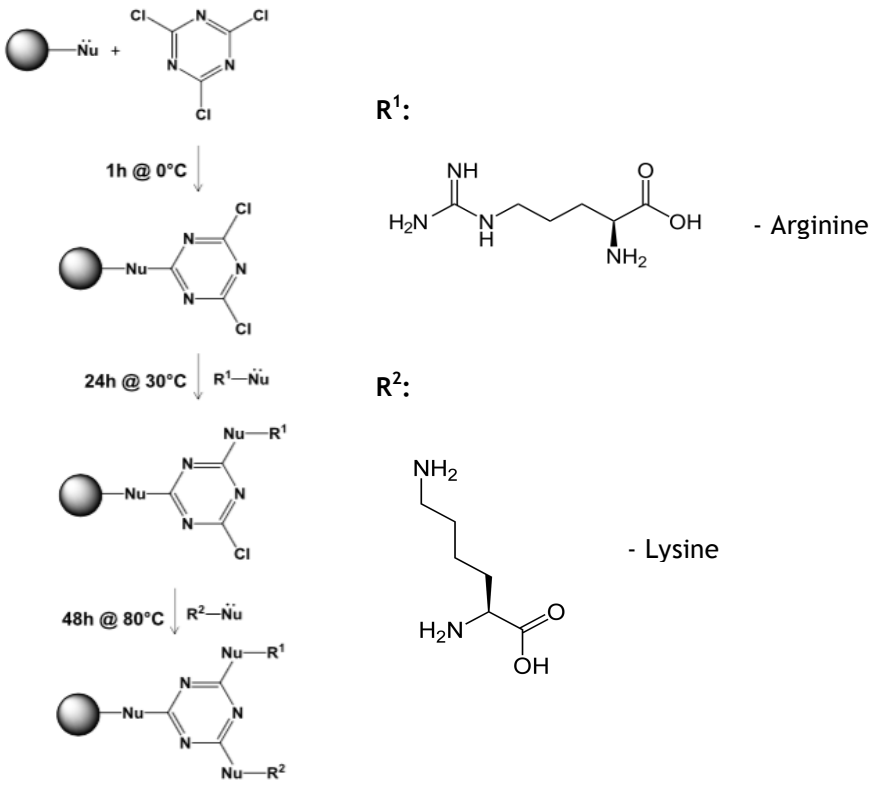


Figure 12. Schematics solid-phase synthesis of triazine-based ligands. A solid matrix functionalized with a nucleophile (denoted by Nü) readily reacts with cyanuric chloride at 0 °C. The cyanuric chloride-modified scaffold undergoes two subsequent nucleophilic substitutions by R^1 and R^2 substituted nucleophiles, where R^1 and R^2 are arginine and lysine, respectively. The first substitution occurs at 30 °C for 24 h and the second substitution at 80 °C for 48 h (adapted from [60]).

3.2.9 Energy dispersive X-ray spectroscopy

In order to confirm if the amino acid ligands (arginine and lysine) were immobilized in the triazine-agarose matrix, an energy dispersive X ray analysis (EDX) was performed in Rontec, and the samples were placed on aluminum stub supports, air-dried at room temperature and finally sputter-coated with gold. It was measured the percentage of different atoms, such as Carbon, Oxygen, Sodium, Phosphorus and Nitrogen present in agarose matrix and triazine-agarose functionalized with arginine and lysine.

3.2.10 Zeta potential measurement

Zeta potential measurement of matrices was performed in a Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK). Before analysis, the matrices were diluted and ressuspended in filtered and deionized water. The surface charges (zeta potential) were assessed in a zeta disposable folded capillary cells and determined by laser Doppler electrophoresis using a Zetasizer Nano ZS (Malvern Instruments Ltd., UK), at 25 ºC. The average values of size and zeta potential were calculated with the data obtained from three measurements ± SD.

3.2.11 Cell culture

HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) High Glucose Medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10 % v/v of fetal bovine serum (FBS) and a mixture of penicillin (100 µg/mL) and streptomycin (100 µg/mL). All cells were grown at 37 ºC in a humidified atmosphere with 5 % of CO_2 in air.

3.2.11.1 Transfection

The purpose of the transfection protocol was to verify the expression of p53 protein with PP-p53 and mcDNA-p53, in order to identify the most effective biomolecule. Thus, the transfection protocol was performed with X-tremeGENE^{TM} HP DNA Transfection Reagent (Roche

Diagnostics GmbH, Germany) according to manufacturer's instructions, in order to transfect HeLa cells with PP-p53 and mcDNA-p53 obtained by NZYTech extraction kit. Briefly, HeLa cells were cultured until a confluence of 80 % to 90 % was obtained. Then, complete medium was substituted by medium without antibiotic and FBS and cells were incubated for 24 hours. Thereafter, K2 transfection reagent and different plasmid samples (1 µg of each vector) were incubated at room temperature for 20 minutes and added to the respective wells. Protein extraction, RNA extraction and immunocytochemistry were performed with non-transfected and transfected cells. Cells intended to be used for protein extraction were cultured in 6-wells and incubated during 72h after transfection, while cells planned to be used for immunocytochemistry were cultured in 12-wells with 15 mm coverslips and incubated during 48 h after transfection and cells used for RNA extraction were cultured in 12-wells and incubated during 24 and 48 h after transfection.

3.2.11.2 RNA extraction

Transfected cells and controls were used for RNA extraction by adding 250 µL of TripleXtractor to each well (12 well plates). The samples were either immediately stored at -80 ºC or used to RNA extraction. The samples were incubated 5 minutes at room temperature, followed by adding 50 µL of chloroform and vigorous mixing, leading to the separation of the different biomolecules present in the sample. Then, incubation was carried out for 10 minutes at room temperature, followed by centrifugation at 4 ºC for 15 minutes at 12000 g in a Hettich Mikro 200R centrifuge (Hettich Andreas GmbH, Tuttlingen, Germany). Thereafter, the aqueous phase was carefully pipetted, to recover all RNA species without destabilizing other biomolecules and contaminating the retrieved sample. RNA was precipitated by adding 125 µL of ice-cold isopropanol, followed by gentle shaking and 10 minutes incubation on ice. Centrifugation was performed for 15 minutes at 4 ºC and 12000 g. The pellet was washed with 125 µL of 75 % Ethanol in Diethylpyrocarbonate (DEPC) water to remove possible organic compounds and centrifuged for 5 minutes at 12000g. Finally, the supernatant was discarded and the pellet was resuspended in 20 µL DEPC water. An electrophoresis in 1 % agarose gel was carried out to confirm the success of the technique. RNA quantification was performed with Nanophotometer (Implen GmbH, Munich, Germany).

3.2.11.3 Protein extraction

Transfected and control cells were also submitted to protein extraction. Briefly, cells were scrapped with cell scrapper and phosphate buffered saline (PBS) and divided in microtubes. Then, the microtubes were centrifuged at 11500 rpm for 7 minutes at 4 ºC in a Hettich Mikro 200R centrifuge (Andreas Hettich GmbH, Tuttlingen, Germany). The supernatant was discarded and 500 µL of PBS were added, followed by centrifugation in the same conditions. Then, supernatant was discarded again and complete lysis medium was added according to pellets volume, followed by 10 minutes of incubation on ice. Complete lysis medium consists in 25 mM Tris (pH 7.4), 2.5 mM EDTA, 2 % Triton X-100, 2.5 mM EGTA, 1 mM PMSF, 10 µL/mL complete EDTA free protease inhibitor cocktail (Roche, Indianapolis, USA). The resulting proteins were used to perform western blot technique.

3.2.12 Immunocytochemistry

Immunocytochemistry was used to evaluate and compare the transfection efficiency of the PP and mcDNA vectors, by assessing the green fluorescent protein (GFP) fluorescence. To perform this technique, it was necessary to place the coverslips previously to the seeding of cells in 12-wells. After 48 hours of transfection, the coverslips were gently washed 3 times with PBS, fixed with 4 % paraformaldehyde for 15 minutes, washed again 3 times with PBS, and permeabilized with 1 % Triton X-100 in PBS for 5 minutes. The coverslips were washed 3 times with PBS 0.1 %, followed by 10 minutes incubation with DAPI for nucleus staining. The coverslips were mounted with a drop of mounting medium and stored at 4 ºC or -20 ºC. Coverslips were analyzed using a Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss SMT, Inc., Oberkochen, Germany) and image achievement was performed with Zeiss Zen software. Immunofluorescence analysis and image manipulation was performed with ImageJ 1.0 software.

3.2.13 Reverse transcription polymerase chain reaction

3.2.13.1 cDNA synthesis

To assess mRNA expression of target proteins it is necessary to synthetize cDNA from the extracted RNA. Thus, 500 ng of total RNA from non-transfected and transfected cells (24 h-48 h post transfection) were reversely transcribed into cDNA with RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Molecular Biology, USA). According to the manufacturer's instructions, 0.5 µL of Random Primers was added, followed by H_2O adding to a final volume of 6 µL. The samples were incubated for 5 minutes at 65 ºC in a thermal cycler T Professional Basic Gradient (Biometra GmbH, Göttingen, Germany). Afterwards, 2 µL of reaction buffer, 0.5 µL of Ribolock RNase Inhibitor, 1 µL dNTP mix and 0.5 µL of Revertaid M-MuPVRT were added by this exact order. Then, cDNA synthesis reaction was incubated for 5 minutes at 25 ºC, followed by 60 minutes at 42 ºC and, finally for 5 min at 70 ºC, in the thermal cycler. Finally, the resultant cDNA was stored at -20 ºC.

3.2.13.2 RT-PCR

Reverse transcription polymerase chain reaction (RT-PCR) was performed by using the thermal cycler Gradient Basic Professional T (Biometra GmbH, Goettingen, Germany). RT-PCR was performed by adding 1 µL cDNA to a reaction with 6.25 µL Taq polymerase, 0.5 µL $MgCl_2$, 0.4 µL of each forward and reverse primer. Primers were designed specifically for p53 gene (Fw: 5'- CCT CAC CAT CAT CAC ACT GG -3'; Rv: 5'- CCT CAT TCA GCT CTC GGA AC -3'), with resulting amplification of a DNA fragment of 286 bp. The following cycle conditions were used: 95 ºC for 5 minutes, 20 cycles at 95 ºC for 30 seconds, 60 ºC for 30 seconds and 72 ºC for 1 minute and final extension at 72 ºC for 5 minutes.

3.2.14 Western Blot

3.2.14.1 Protein quantification

The Bradford micro-assay from BioRad (Hemel Hempstead, UK) was used to measure the protein concentration. In summary, 1 µL of sample, 159 µL of MiliQ water and 40 µL of Biorad reagent were prepared in a microplate for each sample. The assay was performed in triplicate. Upon 15 minutes of incubation at room temperature, absorbance was recorded at 595 nm in an Anthos 2020 microplate reader (Biochrom, Cambridge, United Kingdom). Afterwards, a calibration curve was constructed, by using Bovine Serum Albumin (BSA) from Sigma-Aldrich (St. Louis, MO, United States of America) as a standard protein (0.2-10 µg/µL), as depicted in Figure 13.

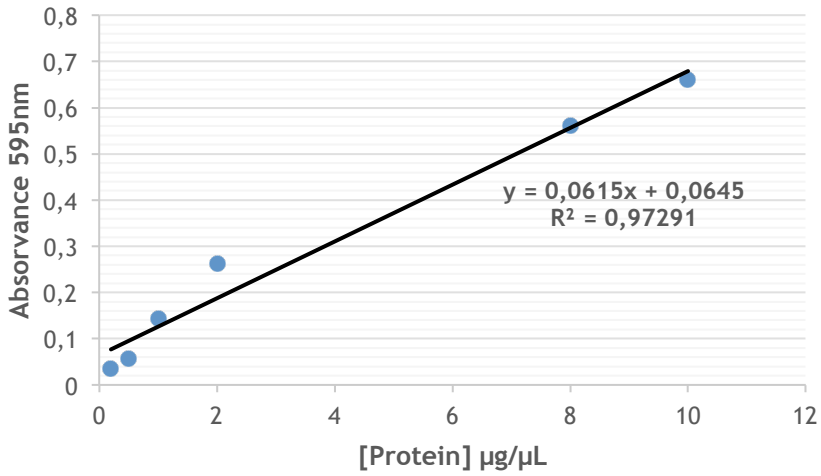


Figure 13. Calibration curve with protein standards (0.2-10 µg/µL).

3.2.14.2 Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed with pooled fractions of proteins of transfected and control cells, using 12.5 % polyacrylamide resolving gel and 4.7 % polyacrylamide stacking gel. The samples were prepared with 25 µg of each protein sample, whose volume was calculated through the calibration curve constructed by protein quantification, and 5 µL of loading buffer, followed by 5 minutes of denaturing at 100 ºC. The

molecular weight marker used was NZYColour Protein Marker II (NZYTech, Lda. - Genes and Enzymes, Lisbon, Portugal). Afterwards the electrophoresis was carried out at 200 V, for 45 minutes. The resulting electrophoresis gel was used for electroblotting.

3.2.14.3 Electroblotting

Protein gel electrotransfer is based on the transference of total proteins of an electrophoresis gel to a polyvinylidenedifluoride membrane. Electroblotting was carried out through a Biorad System with the protein gel resulting from previous electrophoresis. Membrane activation was performed with sheer methanol, followed by MiliQ water equilibration. Electrotransfer was performed at 0.75 mA and 250 V for 90 minutes. Afterwards, the membrane was washed 3 times with Washing Buffer (20mM Tris at pH=8.0, 150mM NaCl, 0.1 % Tween 20) for 5 minutes, followed by 1 hour of incubation at room temperature with milk, under constant agitation. Afterwards, the membrane was washed 3 times for 5 minutes, cut-out and incubated by 1 hour at room temperature with rabit anti-p53 monoclonal antibody (Santa Cruz Biotechonology, Heidelberg, Germany). Hereafter, the membranes were washed 3 times with Washing buffer for 5 minutes and incubated at room temperature with goat anti-rabit IgG polyclonal antibody (Santa Cruz Biotechnology Heidelberg, Germany) for 1 hour, followed by washing 3 times for 5 minutes with Washing buffer. Then, membranes were incubated for 5 minutes with ECL - Westerb blotiing detection kit (WesternBright™ Quantum, Advansta Inc., USA) and were visualized in BioRad ChemiDoc™ Imaging System (Bio-Rad, Hemel Hempstead, UK).

3.2.15 Agarose gel electrophoresis

Samples obtained at the end of the enzymatic digestion, PCR, lysis and in each chromatographic assay were analyzed by horizontal electrophoresis (Clever Scientific) with a 0.8 %/1 % agarose gel with 15 cm length (Hoefer, San Francisco, CA, USA) stained with 0.06 µL of Greensafe. Electrophoresis was run for 30 min at 120 V in TAE buffer (40 mM of Tris base, 20 mM of acetic acid and 1 mM EDTA at pH=8.0). The gel was analyzed under ultraviolet (UV) light using the Uvitec Cambridge Fire-Reader UV system equipped with a camera (UVITEC Cambridge, Cambridge, UK).

CHAPTER IV

Chapter IV – Results and Discussion

Considering the high prevalence of cervical cancer caused by HPV infection in women worldwide, and consequently, the increasing number of deaths caused by this pathology, the development of new therapeutic approaches based on DNA delivery is increasingly required. Plasmid DNA has been the non-viral vector mainly used in DNA therapeutics, but despite some successful cases, it has some obstacles that may be exceeded by using a new promising gene therapy vector, the minicircular DNA.

In this way, the main goals of this thesis are the construction, production and purification of a mcDNA vector encoding p53 to reestablish the levels of this tumor suppressor protein in cervical cancer cells. Thus, in this present work it was firstly constructed the PP-p53 vector, followed by optimization of mcDNA-p53 production. Finally, it was also studied a purification strategy by exploring the simultaneous affinity character of two amino acid ligands immobilized in the same functional group in order to purify the sc mDNA-p53 isoform. At last, it was performed *in vitro* studies in order to evaluate the transfection efficiency, the presence of p53-mRNA transcripts and p53 expression.

4.1 mcDNA-p53 Construction

4.1.1 pDNA-FLAG-p53 and Parental Plasmid Amplification and Recovery

The production and recovery of high-copy number of sc pDNA is fundamental for therapeutic applications. Some factors such as the host, the recombinant plasmid vector and growth conditions must be taken into account to obtain an efficient manufacturing process, which can provide large quantities of plasmid production [61]. *Escherichia coli* strains have been considered the most efficient host for pDNA production, not only yielding high amounts of vector but also maintaining its integrity [36].

In order to obtain the p53 fragment, it was required the pDNA-FLAG-p53 amplification. This vector was obtained by *E. coli* DH5*α* fermentation in a refrigerated orbital at 37 ºC, and with agitation of 250 rpm until it reaches an OD_{600nm} of 8. In the first step, a pDNA-FLAG-p53 plasmid sample was obtained with NZYTech kit, using the NZYTech protocol without modifications, and the resultant sample was analyzed by horizontal electrophoresis with a 0.8 %

agarose gel stained with 0.06 µL of GreenSafe (Figure 14A). On the other hand, to obtain the vector's base for mcDNA-p53 construction, Parental Plasmid amplification was obtained by *E. coli* ZYCY10P3S2T fermentation in an orbital at 42 ºC, and with agitation of 250 rpm until it reaches an OD_{600nm} of 5. In the next step, PP sample was obtained with NZYTech kit, using the NZYTech protocol with the same modifications adopted for mcDNA extraction, as described in section 3.2.9, in order to maintain the samples at the same conditions. These conditions of PP production and recovery were already described in a previous study [53]. Finally, PP sample was analyzed by horizontal electrophoresis with a 0.8 % agarose gel stained with 0.06 µL of GreenSafe (Figure 14B). It can be concluded that after DNA extraction the resultant samples mainly contain the sc isoform pDNA-FLAG-p53 and sc PP, but still containing the open circular isoform as contaminant.

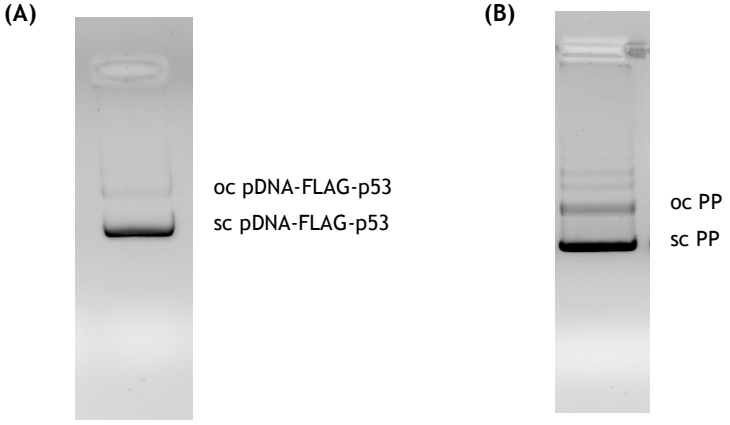
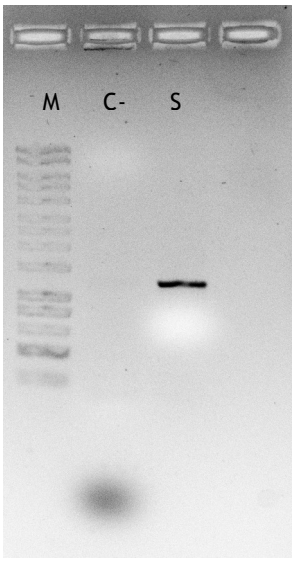


Figure 14. (A) Electrophoresis of *E. coli* DH5*α* cell lysates: pDNA sample purified by NZYTech purification kit. **(B)** Agarose gel electrophoresis of *E. coli* ZYCY10P3S2T cell lysates: PP sample purified by NZYTech purification modified kit.

4.1.2 p53 Fragment Amplification

After pDNA-FLAG-p53 isolation, PCR amplification was required in order to obtain the p53 fragment. The amplification was carried out by using specific primers that were designed to amplify the p53 gene and including the ends of sequence for the restriction enzymes recognition: XbaI and BamHI represented in section 3.2.2.

The amplified p53 fragment was purified by PCR Purification Kit, and analyzed by 1 % agarose gel electrophoresis (Figure 15). As depicted in Figure 15, it can be observed a single band with approximately 1.2 kbp that correspond to the size of p53 gene fragment. Thus, this result shows that amplification has successfully occurred.



p53 fragment (1.2kbp)

Figure 15. Agarose gel electrophoresis of PCR amplification. M - Molecular weight marker, C- -Negative control of PCR amplification, S - p53 fragment amplified by PCR and purified by PCR purification kit.

4.1.3 p53 Fragment and PP Digestions

In order to proceed with the cloning step, it was necessary to use the same restriction enzymes on both p53 fragment and PP vector. For that, it was used two restriction enzymes: XbaI and BamHI that will recognize their restriction sites present in both p53 fragment and PP Multiple Cloning Site (MCS) (Figure 16).

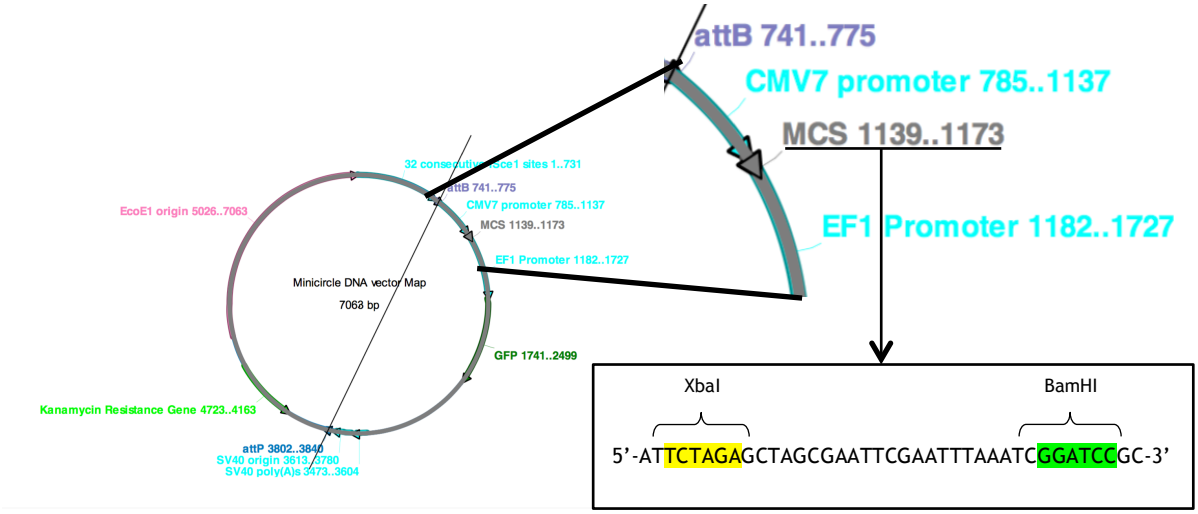


Figure 16. XbaI and BamHI restriction enzymes sites in PP map.

Firstly, it was confirmed if PP vector's linearization completely occurred by using one of the restriction enzymes. Thus, the enzymatic digestion was performed with XbaI during 1 hour at 37 ºC, and then, the digestion product was purified with PCR purification kit and analyzed by 0.8 % agarose gel electrophoresis (Figure 17). The results proved that the digestion was successful, being present a single band corresponding to a linear PP. Simultaneously, the p53 fragment amplified before, was also digested with XbaI in the same conditions, but was not analyzed by agarose gel electrophoresis due to the fact that it will not occur any difference in the molecular size of fragment after digestion. This sample was also purified with the PCR purification kit for further digestion with the second enzyme.

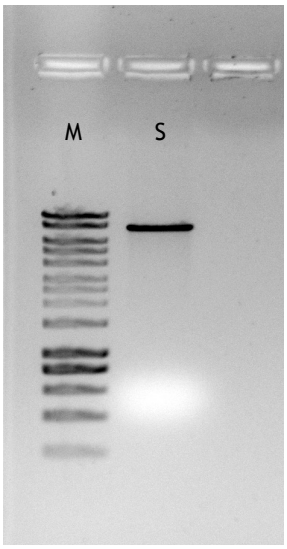


Figure 17. Agarose gel electrophoresis of PP linearization. M – Molecular weight marker, S – PP vector digested with XbaI and purified by PCR purification kit.

After digestion of PP and p53 fragment with XbaI, it was performed the enzymatic digestion with the second enzyme, BamHI, during 1 hour at 37 ºC, and then, the samples were purified with the PCR purification kit and analyzed by 1 % agarose gel electrophoresis. The result presented in Figure 18 shows the successful digestion of PP vector, being observed only one band correspondent to the linear fragment of 7.06 kbp from this vector. In addition, it is also confirmed the presence of p53 fragment, due to the band that arises at 1.2 kbp (by comparing to the molecular weight marker). By this result, it is also possible verify that, both fragments present a good integrity to proceed the cloning process.

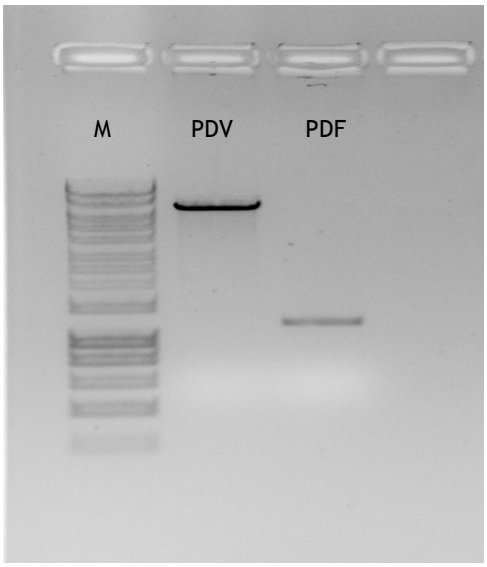


Figure 18. Agarose gel electrophoresis of PP and p53 fragment digestions. M - Molecular weight marker; PDV – PP vector digested with XbaI and BamHI, and purified by PCR purification kit; PDF – p53 fragment digested with XbaI and BamHI, and purified by PCR purification kit.

4.1.4 Cloning and *E. coli* Transformation

After digestions and purification of PP vector and p53 fragment, the fragments acquired the same cohesive ends due to restriction enzymes. Thus, it was proceeded to the cloning step that consisted in ligation of p53 fragment into PP vector. Several ligation conditions were tested such as different ratios of PP vector/p53 fragment (1/3, 3/1 and 1/1), diverse incubation times and different incubation temperatures (overnight at 4 ºC, 3 h at 16 ºC/21 ºC). It was still tested the previous cloning conditions by using dephosphorylated PP vector or non-dephosphorylated PP vector, due to the fact that dephosphorylated vector can prevent and possibly stop the self-ligation of vector during cloning step.

Subsequently, *E. coli* Top10 strain was transformed with previous cloning products by heat shock, because this strain is more suitable for large plasmid transformation [62, 63]. Isolated colonies were replicated to another agar plate and, at the same time, resuspended in 10 µL of 20 mM NaOH, lysed at 100 ºC for 5 minutes and used for PCR test to confirm p53 fragment presence. After this, samples were analyzed by 1% agarose gel electrophoresis (Figure 19).

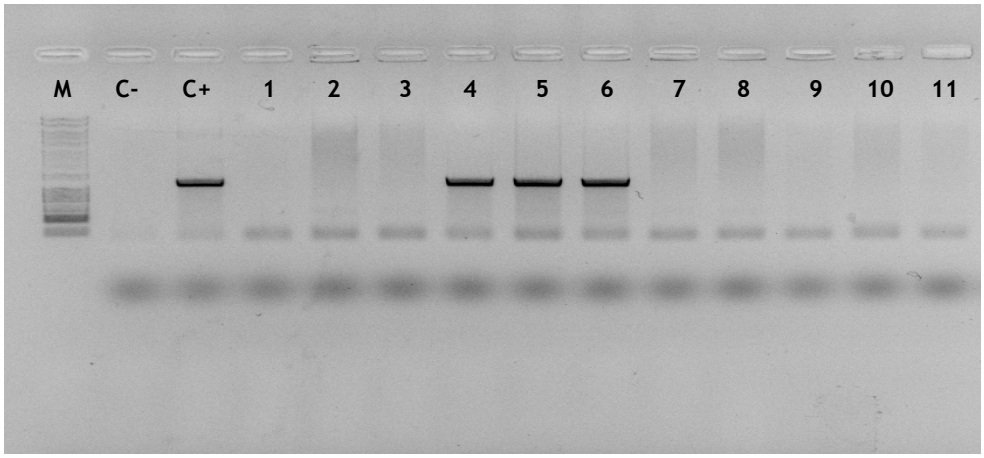
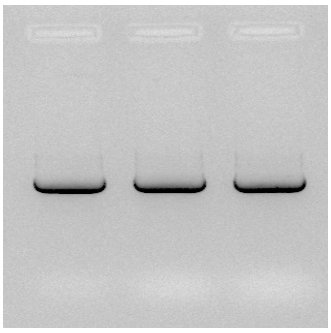


Figure 19. Agarose gel electrophoresis of colonies PCR. M - Molecular weight marker; C- - Negative control of PCR; C+ - Positive control of PCR; Lane 1-3 – PCR amplification of colonies from 1/3D ratio; Lane 4-7 - PCR amplification of colonies from 3/1D ratio; Lane 8-11 – PCR amplification of colonies from 1/3 ratio.

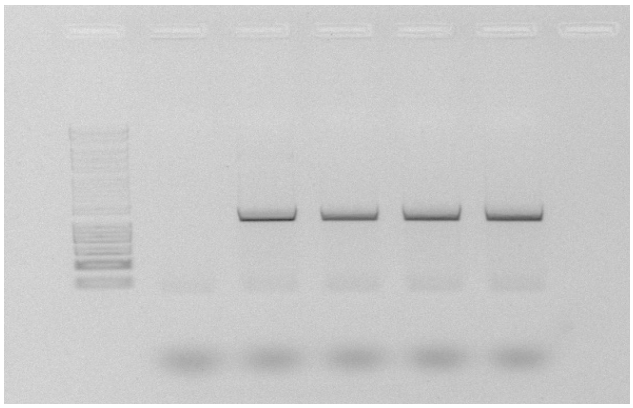
In Figure 19, PCR positive control was made by using the pDNA-FLAG-p53 vector. The PP-p53 transformation occurred only in 3/1D ratio of insert/dephosphorylated vector, represented by the lanes 4, 5 and 6. However, results from the ratio 1/1 were not shown because it was not possible isolate colonies from the agar plate. After confirming the presence of the p53 gene in the PP vector of transformed colonies, these colonies were placed to grow in 3 mL of LB during 12 hours at 37 ºC with agitation of 250 rpm. PP-p53 was isolated by miniprep extraction, used for a final PCR test to confirm the integrity and presence of p53 fragment in the PP-p53 vector, and both assays were analyzed by 0.8 % agarose gel electrophoresis, before being used in next steps (Figure 20).

(A)



sc PP-p53 (8.26 kbp)

(B)



p53 fragment (1.2 kbp)

Figure 20. (A) Agarose gel electrophoresis of PP-p53 miniprep extraction; Lane 4-6 - PP-p53 miniprep extraction corresponding to the colonies 4,5,6 respectively. **(B)** Agarose gel electrophoresis of PP-p53 PCR. M - Molecular weight marker; C- - Negative control of PCR; C+ - Positive control of PCR; Lane 4-6 – PCR amplification of PP-p53 miniprep extraction.

Since the 3 PP-p53 vectors presented the fragment corresponding to the p53 gene, only the PP-p53 corresponding to colony 4 was sequenced. Three sequencings were performed by STABVIDA: two with the primers designed to recognize the p53 encoding gene, previously used, and one with the commercial primer that recognize the CMV site present in the PP (Table 9) (Figure 21).

Table 9. Primer for PP-p53 sequencing.

Primer	
CMV Fw	5'-CGC AAA TGG GCG GTA GGC GTG-3'

Score	Expect	Identities	Gaps	Strand
1537 bits(832)	0.0	832/832(100%)	0/832(0%)	Plus/Plus

```
Query  936   GAGGAGCCGCAGTCAGATCCTAGCGTCGAGCCCCCTCTGAGTCAGGAAACATTTTCAGAC  995
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  77    GAGGAGCCGCAGTCAGATCCTAGCGTCGAGCCCCCTCTGAGTCAGGAAACATTTTCAGAC  136

Query  996   CTATGGAAACTACTTCCTGAAAACAACGTTCTGTCCCCCTTGCCGTCCCAAGCAATGGAT  1055
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  137   CTATGGAAACTACTTCCTGAAAACAACGTTCTGTCCCCCTTGCCGTCCCAAGCAATGGAT  196

Query  1056  GATTTGATGCTGTCCCCGGACGATATTGAACAATGGTTCACTGAAGACCCAGGTCCAGAT  1115
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  197   GATTTGATGCTGTCCCCGGACGATATTGAACAATGGTTCACTGAAGACCCAGGTCCAGAT  256

Query  1116  GAAGCTCCCAGAATGCCAGAGGCTGCTCCCCCCGTGGCCCCTGCACCAGCAGCTCCTACA  1175
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  257   GAAGCTCCCAGAATGCCAGAGGCTGCTCCCCCCGTGGCCCCTGCACCAGCAGCTCCTACA  316

Query  1176  CCGGCGGCCCCTGCACCAGCCCCCTCCTGGCCCCTGTCATCTTCTGTCCCTTCCCAGAAA  1235
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  317   CCGGCGGCCCCTGCACCAGCCCCCTCCTGGCCCCTGTCATCTTCTGTCCCTTCCCAGAAA  376

Query  1236  ACCTACCAGGGCAGCTACGGTTTCCGTCTGGGCTTCTTGCATTCTGGGACAGCCAAGTCT  1295
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  377   ACCTACCAGGGCAGCTACGGTTTCCGTCTGGGCTTCTTGCATTCTGGGACAGCCAAGTCT  436

Query  1296  GTGACTTGCACGTACTCCCCTGCCCTCAACAAGATGTTTTGCCAACTGGCCAAGACCTGC  1355
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  437   GTGACTTGCACGTACTCCCCTGCCCTCAACAAGATGTTTTGCCAACTGGCCAAGACCTGC  496

Query  1356  CCTGTGCAGCTGTGGGTTGATTCCACACCCCCGCCCGGCACCCGCGTCCGCGCCATGGCC  1415
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  497   CCTGTGCAGCTGTGGGTTGATTCCACACCCCCGCCCGGCACCCGCGTCCGCGCCATGGCC  556

Query  1416  ATCTACAAGCAGTCACAGCACATGACGGAGGTTGTGAGGCGCTGCCCCCACCATGAGCGC  1475
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  557   ATCTACAAGCAGTCACAGCACATGACGGAGGTTGTGAGGCGCTGCCCCCACCATGAGCGC  616

Query  1476  TGCTCAGATAGCGATGGTCTGGCCCCTCCTCAGCATCTTATCCGAGTGGAAGGAAATTTG  1535
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  617   TGCTCAGATAGCGATGGTCTGGCCCCTCCTCAGCATCTTATCCGAGTGGAAGGAAATTTG  676

Query  1536  CGTGTGGAGTATTTGGATGACAGAAACACTTTTCGACATAGTGTGGTGGTGCCCTATGAG  1595
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  677   CGTGTGGAGTATTTGGATGACAGAAACACTTTTCGACATAGTGTGGTGGTGCCCTATGAG  736

Query  1596  CCGCCTGAGGTTGGCTCTGACTGTACCACCATCCACTACAACTACATGTGTAACAGTTCC  1655
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  737   CCGCCTGAGGTTGGCTCTGACTGTACCACCATCCACTACAACTACATGTGTAACAGTTCC  796

Query  1656  TGCATGGGCGGCATGAACCGGAGGCCCATCCTCACCATCATCACACTGGAAGACTCCAGT  1715
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  797   TGCATGGGCGGCATGAACCGGAGGCCCATCCTCACCATCATCACACTGGAAGACTCCAGT  856

Query  1716  GGTAATCTACTGGGACGGAACAGCTTTGAGGTGCGTGTTTGTGCCTGTCCTG  1767
             ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  857   GGTAATCTACTGGGACGGAACAGCTTTGAGGTGCGTGTTTGTGCCTGTCCTG  908
```

Figure 21. DNA sequence alignment of p53 gene and p53-cloned PP.

By analysis of sequencing results, it was conclued that PP-p53 contains the coding sequence of p53 tumor suppressor protein. So, *E. coli* ZYCY10P3S2T strain was transformed with this PP-p53 vector by heat shock, because this strain allows the plasmid recombination through the activation of *serine phiC31 recombinase* that acts on the attB and attP sites to form mcDNA and miniplasmid species, after induction with L-arabinose during cell growth [52]. Also in this case, isolated colonies were replicated to another agar plate and, at the same time, resuspended in 10 µL of 20mM NaOH, lysed at 100 ºC for 5 minutes and used for PCR test to confirm p53 fragment presence. After this, samples were analyzed by 0.8 % agarose gel electrophoresis (Figure 22).

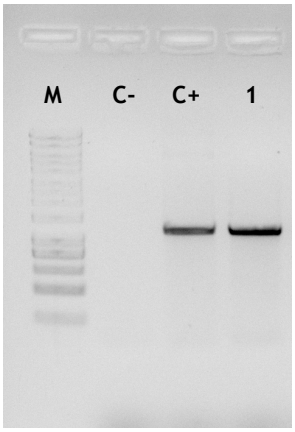


Figure 22. Agarose gel electrophoresis of colony PCR. M - Molecular weight marker; C- - Negative control of PCR; C+ - Positive control of PCR; Lane 1 – PCR amplification of colony.

In Figure 22, the pDNA-FLAG-p53 vector was used as PCR positive control, and one transformed colony was tested by PCR and it was confirmed the presence of the p53 fragment. After this, it was created and cryopreserved bacterial banks with this strain of *E. coli* containing the PP-p53, for further production studies.

4.2 Optimization of mcDNA-p53 Production

As mentioned before, *E. coli* ZYCY10P3S2T strain allows the parental plasmid recombination to form mcDNA and miniplasmid species by induction with L-arabinose during cell growth. Therefore, it was initially considered that it was important to test the conditions already established for another vector, where it was studied the intramolecular recombination promoted by using two L-arabinose percentages (0.01 % and 0.1 %), in which the content of mcDNA and contaminants (mP and PP residues) were evaluated along 5 hours. So, the conditions applied for induction of mcDNA were a temperature of 32 ºC during 3 hours, with agitation of 250 rpm. Thus, after the initial PP production stage (250 rpm at 42 ºC), the mcDNA production process was started with addition of 125 mL of inducer mixture composed by 25 g/L LB medium and 1.0 M NaOH at pH=7, into a 125 mL of medium fermentation. To this mixture it was also added an inducer, 0.01 % L-arabinose to promote the serine recombinase phiC31 expression, allowing the production of mcDNA and mP. Simultaneously, to promote the elimination of residual mP and PP, the I-SceI endonuclease cleaves specific sites in these biomolecules.

Another important strategy to facilitate the induction step is measuring the OD_{600nm} from hour to hour during the fermentation, in order to guarantee the beginning of the

induction at the most appropriate moment, which is at the end of the log phase, when there is a higher cell density associated with less metabolic stress.

Subsequently, the mcDNA sample was recovered and obtained with a modified NZYTech kit, described in 4.3 and sample was analyzed by horizontal electrophoresis by using 0.8 % agarose gel stained with 0.06 µL of GreenSafe (Figure 23).

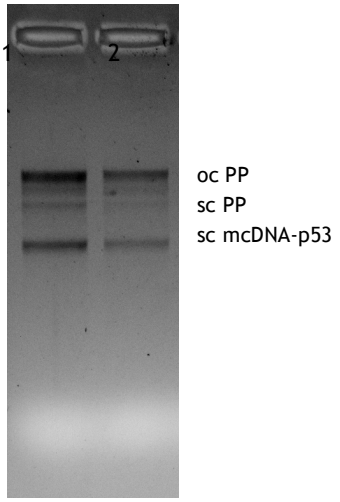


Figure 23. Agarose gel electrophoresis of mixture of nucleic acids maxiprep extraction: mcDNA samples 1 and 2 purified by NZYTech purification modified kit.

The existence of high quantity of PP in the sample is a consequence of a less efficient recombination that in turn leads to a low production of mcDNA, such as it is observed in Figure 23. Comparing with the mcDNA induction already described, this can occur given that the PP-p53 vector increased of size due to the presence of 1200 bases of p53 gene, and taking into account that the goal was to produce large amounts of mcDNA-p53, it was required to perform an optimization in the induction process.

In order to improve the yield of mcDNA obtained after induction, which it will have a significant impact on the downstream process, other induction conditions were also tested: the inductor concentration and time of induction. In the literature there are several recommended percentages of inductor, such as 0.01 % [53] and 2 % [64], thus, in this work, three concentrations of L-arabinose were also tested: 0.001 %, 0.01 % and 0.1 %, in order to try a lower concentration and a higher concentration compared to that already tested previously. At the same time, different induction times were also tested: 2, 3 and 4 hours. With the studied conditions, it could be observed whether the concentration and time initially tested was insufficient to promote efficient recombination or whether it would be excessive and could carry some cellular toxicity. Thus, the samples (containing mcDNA and PP species) were recovered and obtained with a modified NZYTech kit in the end of induction, and analyzed by horizontal electrophoresis using 0.8 % agarose gel stained with 0.06 µL of GreenSafe. It was

injected the same amount of sample (2µg) in all wells of agarose gel electrophoresis (Figure 24).

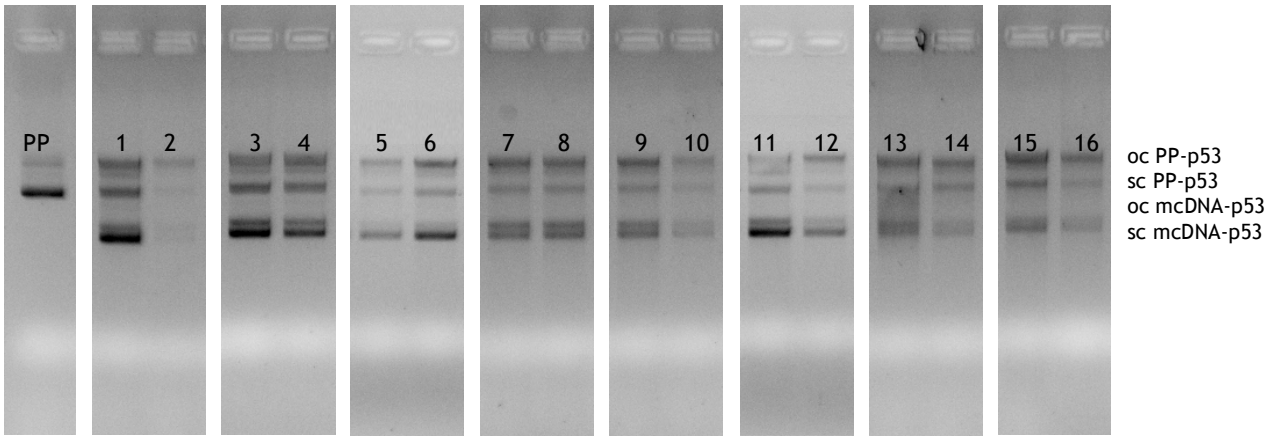


Figure 24. Agarose gel electrophoresis of mixture of nucleic acids maxiprep extraction. PP – PP control sample; Lane 1, 2 – maxiprep extraction after 2 h of induction with 0.001 % of L-arabinose; Lane 3, 4 – maxiprep extraction after 2h of induction with 0.01% of L-arabinose; Lane 5, 6 – maxiprep extraction after 2 h of induction with 0.1 % of L-arabinose; Lane 7, 8 – maxiprep extraction after 3 h of induction with 0.001 % of L-arabinose; Lane 9, 10 – maxiprep extraction after 3h of induction with 0.01 % of L-arabinose; Lane 11, 12 – maxiprep extraction after 3 h of induction with 0.1 % of L-arabinose; Lane 13, 14 – maxiprep extraction after 4h of induction with 0.01 % of L-arabinose; Lane 15, 16 – maxiprep extraction after 4 h of induction with 0.1 % of L-arabinose.

From maxiprep extraction after 2 h of induction with 0.001 % of L-arabinose represented by the lane 1 and 2 it was obtained good efficiency of recombination (lane 1) but it was not reproducible (lane 2). In the same way, after 3 h of induction with 0.1 % of L-arabinose, it was obtained good efficiency of recombination (lane 11) but it was not reproducible (lane 12). It was achieved a good recombination efficiency by using 2 h of induction with 0.01 % of L-arabinose, represented by lane 3 and 4. A low recombination efficiency was obtained when it was used 2 h of induction with 0.1 % of L-arabinose (lane 5 and 6), 3h of induction with 0.001 % of L-arabinose (lane 7 and 8), 3h of induction with 0.01 % of L-arabinose (lane 9 and 10), 4 h of induction with 0.01 % of L-arabinose (lane 13 and 14), and, and finally, 4 h of induction with 0.1 % of L-arabinose (lane 15 and 16). In general, it can be concluded that for lower concentrations of L-arabinose there was low recombination efficiency at different induction times. However, the mcDNA sample represented by lane 1 showed good efficiency but was not reproducible. This may suggest that this concentration was insufficient to promote efficient recombination. On the other hand, it can be concluded that for higher concentrations of L-arabinose there was also low recombination efficiency at different induction times. Nevertheless, the mcDNA sample represented by lane 11 showed good

efficiency but was not reproducible which may be due to a possible cellular toxicity that the inducer performs and/or due to saturation of the cellular receptors. The intermediate concentration of inductor L-arabinose has shown the best recombination efficiency with reduces the induction time.

Briefly, the best results of mcDNA production were obtained through 2 h of induction with 0.01 % of L-arabinose. The conditions applied have advantages compared to other studies. Namely the use of a lower concentration of inducer, in this case 0.01 % of L-arabinose, makes the induction process cost-effective and more advantageous for industrial application, compared to the 1% and 2% used in a previous studies [65, 64]. On the other hand, by using lower induction times provides advantages by reducing process time to obtain the target biomolecule, which is an important characteristic to apply this process at industrial scale. In this case, when it was used 2 h of induction, it was reduced to half of time applied in other studies. The used conditions are associated with higher yields of mcDNA accompanied by fewer contaminants, namely PP. However, the complexity of the sample obtained at the end of induction is one of the factors that difficult the mcDNA purification.

4.3 mcDNA-p53 Purification

In order to be possible the application of the mcDNA-p53 vector into a therapeutic approach, it is necessary to purify the sc isoform, due to the fact that this isoform present the highest bioactivity. For that, it was studied a new chromatographic column in order to explore the simultaneous affinity character of two amino acid ligands (arginine and lysine) immobilized in the same functional group (triazine) under ionic and hydrophobic binding/elution conditions. This agarose column functionalized with triazine has already been studied but with another purpose of application, namely, the recombinant proteins purification [67, 68]. This matrix, being a functionalized agarose with triazine, brings some advantages, due to the fact that matrix functionalization with two different amino acids that allows exploiting different type of combinations. With these combinations it is possible to study diverse interactions between the target biomolecule and the column. These columns also arise interest because they present fast and simple manufacture and, one of the most important characteristic, is that they are much more cost-effective. Thus, the chromatographic matrix preparation was performed according to the protocol already described in 3.2.11.

After the ligands immobilization, the matrix was characterized by energy dispersive X-ray spectroscopy (EDX) and zeta potential. It was evaluated the percentage of different atomic elements, such as Carbon, Oxygen and Nitrogen by EDX in order to compare the agarose matrix and triazine-agarose matrix functionalized with arginine and lysine. It was obtained a percentage of 54.72 % of Carbon and 43.52 % of Oxygen for the agarose matrix (Table 10) and

for the triazine-agarose functionalized with the amino acids was obtained 50.73 % of Carbon, 43.20 % of Oxygen and 6.07 % of Nitrogen (Table 11).

Table 10. Agarose matrix analysed by energy dispersive X-ray spectroscopy.

Element	C Normal [wt.%]	C Atomic [at.%]	Error (2 Sigma) [wt.%]
Carbon	46.99	54.72	11.19
Oxygen	49.78	43.52	12.05
Sodium	1.92	1.17	0.31
Phosphorus	1.31	0.59	0,16
Total:	100.00	100.00	

Table 11. Triazine-agarose matrix functionalized with arginine and lysine amino acids analysed by energy dispersive X-ray spectroscopy.

Element	C Normal [wt.%]	C Atomic [at.%]	Error (2 Sigma) [wt.%]
Carbon	43.98	50.73	9.49
Oxygen	49.88	43.20	10.87
Nitrogen	6.14	6.07	1.76
Total:	100.00	100.00	

Through EDX results, it was concluded that the functionalization was successful due to the fact that functionalized matrix contains nitrogen atoms associated with the amines of amino acid ligands. On the other hand, comparing the result concerning the agarose matrix with the functionalized matrix, the amount of carbon and oxygen atoms remains constant, taking into account the associated errors.

The matrix was also analyzed by zeta potential to verify the overall load on the surface of both matrices, the functionalized and the non-functionalized. For the agarose matrix was obtained a zeta potential of -0,523, and for the triazine-agarose functionalized with arginine and lysine was obtained a zeta potential of -16,225 (Table 12).

Table 12. Zeta potential measurment of different matrices.

	Zeta Potential				
	1	**2**	**3**	**4**	$\bar{X}$
Agarose Matrix	-0,475	-0,571	-	-	-0,523
Triazine-Agarose functionalized with Arginine and Lysine	-16,4	-13	-18,2	-17,3	-16,225

The results obtained in the Zeta potential shows that the agarose without functionalization did not present an effective superficial charge, being the obtained values near zero. However, the triazine-agarose functionalized with arginine and lysine ligands obtained a negative zeta potential, possibly due to the fact of triazine contains an aromatic ring, which can confer this overall result of charges even though arginine and lysine being two positive amino acids [60].

For the chromatographic tests, a pre-purified mcDNA-p53 sample was used to give an idea of the retention and elution profile of various components present in the sample. Firstly, the binding and elution profile of the column was evaluated by using conditions that mainly promote ionic or hydrophobic interactions. The chromatographic assays were performed in this functionalized triazine-agarose column, with flow rate of 1.0 mL/min. In the study of ionic conditions, the column was equilibrated with 10mM Tris buffer and after injection of 200 µL of sample occurred the elution of a peak in the flowthgrough. Subsequently, an elution step was performed with a stepwise gradient by increasing salt to 2 M NaCl at pH 8.0, and a second peak was eluted (Figure 25A). By the analysis of the agarose gel electrophoresis of the fractions collected from both peaks it was verified that the first peak corresponded to the mcDNA-p53 lysate elution and the second peak was the effect of salt increasing or the effect of impurities such as proteins and endotoxins (Figure 25B). On the other hand, in the study of hydrophobic conditions, the column was equilibrated with 3 M $(NH_4)_2SO_4$ and after injection of 200 µL of sample, occurred the elution of a small peak in the flowthgrough. Subsequently, an elution step was performed with a stepwise gradient by decreasing the ionic strength to 10 mM Tris buffer at pH 8.0, and a second peak was eluted (Figure 25C). By the analysis of the agarose gel electrophoresis of the fractions collected from both peaks, it was verified that the first peak corresponded to the ammonium sulfate presence or, as before, the effect of impurities and the second peak was the mcDNA-p53 lysate elution (Figure 25D).

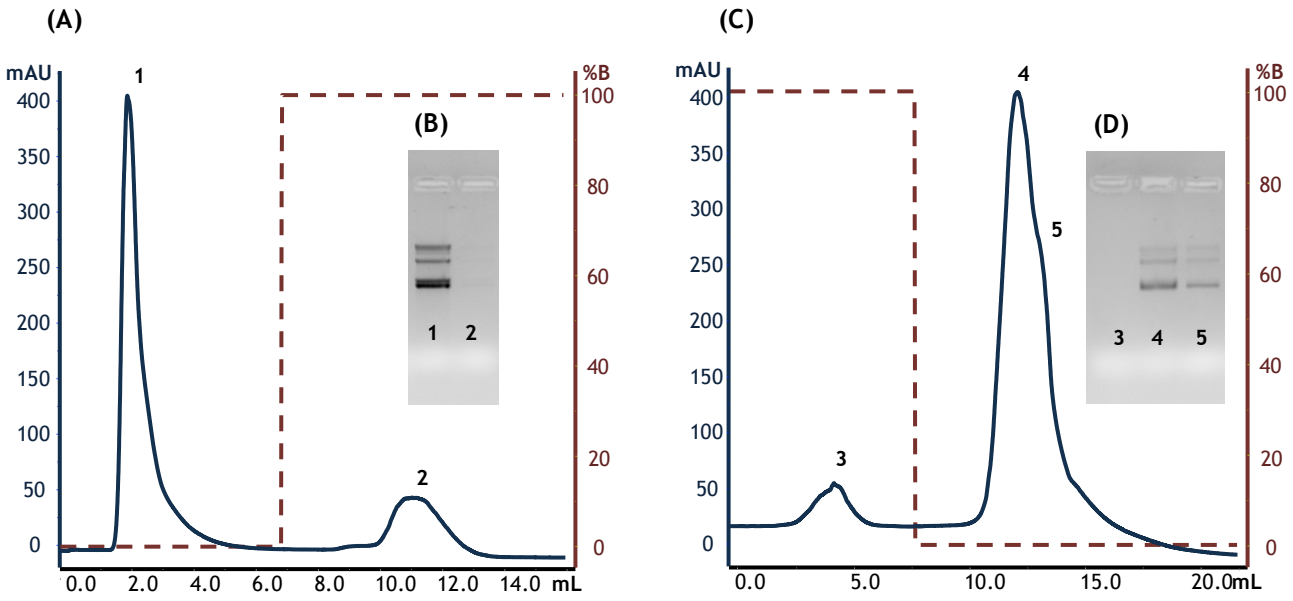


Figure 25. (A) Chromatographic profile of ionic conditions test. Elution was performed at 1.0mL/min by applying a NaCl stepwise gradient from 10mM Tris buffer to 2 M NaCl (pH 8.0), as represented by the % B. **(B)** Agarose gel electrophoresis of samples collected at the column outlet. Fractions corresponding to peaks (1) and (2) are shown in lanes 1 and 2, respectively. **(C)** Chromatographic profile of hydrophobic conditions test. Elution was performed at 1.0 mL/min by decreasing $(NH_4)_2SO_4$ stepwise gradient from 3 M $(NH_4)_2SO_4$ to 10 mM Tris buffer (pH 8.0), as represented by the % B. **(D)** Agarose gel electrophoresis of samples collected at the column outlet. Fractions corresponding to peaks (3), (4) and (5) are shown in lanes 3-5, respectively.

The results of agarose gel electrophoresis showed that for the test of ionic condition, the mcDNA sample did not bind to the matrix with Tris buffer, but using the hydrophobic condition assay the mcDNA sample was bound to the matrix with 3 M $(NH_4)_2SO_4$ and was eluted with Tris buffer. Thus, as in the first case, the sample did not bind to the matrix it can be concluded that there is not much influence of electrostatic interactions for the binding conditions studied. On the other hand, the results indicate the involvement of hydrophobic interactions between the species present in the sample and the ligands of the matrix because only when ammonium sulfate was applied in the equilibrium buffer, it was observed the sample binding to the matrix. Therefore, these interactions were weakened when Tris buffer was used, being promoted the elution and recovery of the species retained in the column.

Arginine and lysine ligands have been already studied in the purification of pDNA by affinity chromatography, being observed mainly the involvement of electrostatic interactions [69, 70]. However, the versatility of these ligands was further explored for the isolation of microRNAs through different elution strategies, among which successfully ammonium sulfate was applied to promote the retention and elution of the different species present in the

sample. A similar behavior was observed in the present work when ammonium sulfate was used, suggesting the involvement of hydrophobic interactions with the aliphatic chains of arginine and lysine amino acids [60,61]. In addition, the aromatic ring of triazine group can establish π-π stacking interactions with the nucleic acids bases [71, 72]. Moreover, the absence of electrostatic interactions and the prevalence of hydrophobic interactions under the binding studied conditions can also be justified by the zeta potential results that showed a negative overall charge of the functionalized matrix, which can cause repulsion by nucleic acids.

Therefore, several elution gradients with ammonium sulfate were tested in order to improve the mcDNA isolation. Initially, it was performed the total mcDNA sample binding into the column, followed by elution and recovery of different components present in the sample. So, the chromatographic assay was accomplished at a flow rate of 1.0 mL/min, the column was equilibrated with 3 M $(NH_4)_2SO_4$ and after the injection of 200 µL of sample the binding/elution of different biomolecules occurred. For the elution step, a decreasing stepwise gradient was applied to 1.5 M $(NH_4)_2SO_4$, and to 10 mM Tris buffer at pH 8.0 (Figure 26A). One more time, samples collected at the column outlet were analyzed by 0.8 % agarose gel electrophoresis. (Figure 26B).

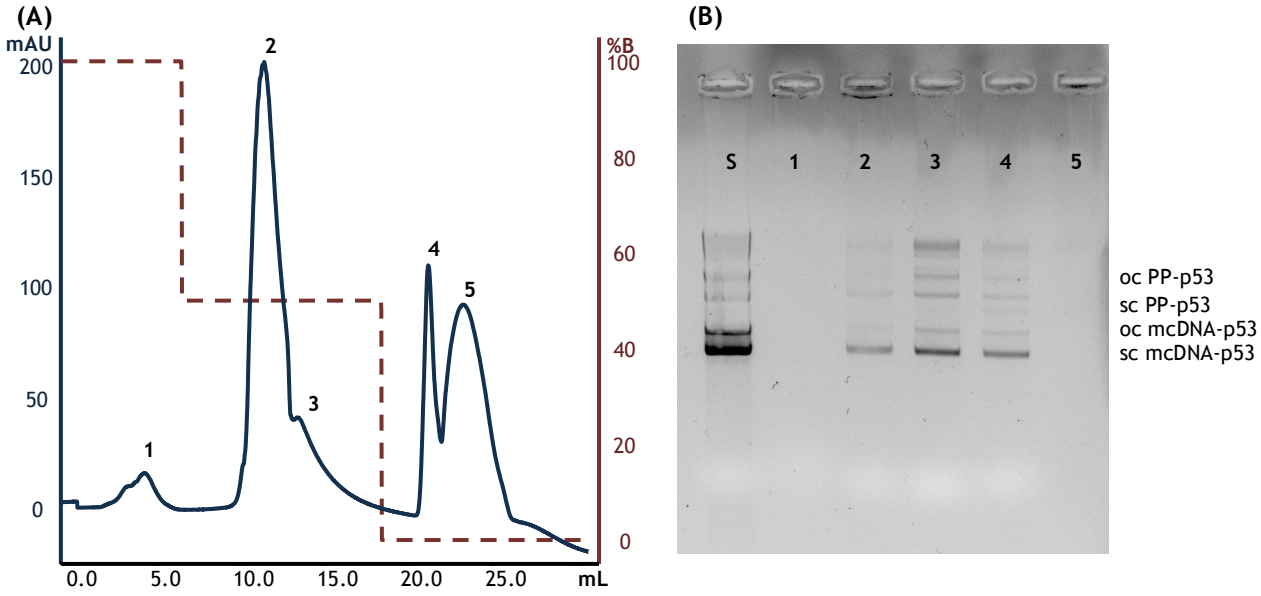


Figure 26. **(A)** Chromatographic profile of lysate using hydrophobic conditions. Elution was performed at 1.0 mL/min by decreasing stepwise gradient from 3 M $(NH_4)_2SO_4$ to 1.5M $(NH_4)_2SO_4$, and finally to 10 mM Tris buffer (pH 8.0), as represented by the % B. **(B)** Agarose gel electrophoresis of samples collected at the column outlet. Fractions corresponding to peaks (1)-(5) are shown in lanes 1-5, respectively. Lane S, mcDNA-p53 sample injected onto the column.

In the previous result, we can observe that the sample completely bound to the column with 3 M of ammonium sulfate. However, the decrease of salt concentration in stepwise gradient did not allow good selectivity to isolate the mcDNA, being present in peaks 2, 3 and 4 eluted at different salt concentrations. Thus, in order to obtain the selectivity between PP and mcDNA several tests were carried out. Taking into account the previous result, it was tested a decreasing salt concentration in the binding step to 2.4 M $(NH_4)_2SO_4$, still maintaining the same procedure with stepwise gradient for half concentration of salt (1.2M $(NH_4)_2SO_4$) at a flow rate of 1.0 mL/min. So, after the column equilibration with 2.4 M $(NH_4)_2SO_4$, 200 µL of sample was injected and a first peak was obtained during the flowthrough. The bound species were eluted in two peaks by decreasing the salt concentration to 1.2 M $(NH_4)_2SO_4$ and 10 mM Tris buffer at pH 8.0 (Figure 27A). Likewise, samples collected at the column outlet were analyzed by 0.8 % agarose gel electrophoresis (Figure 27B).

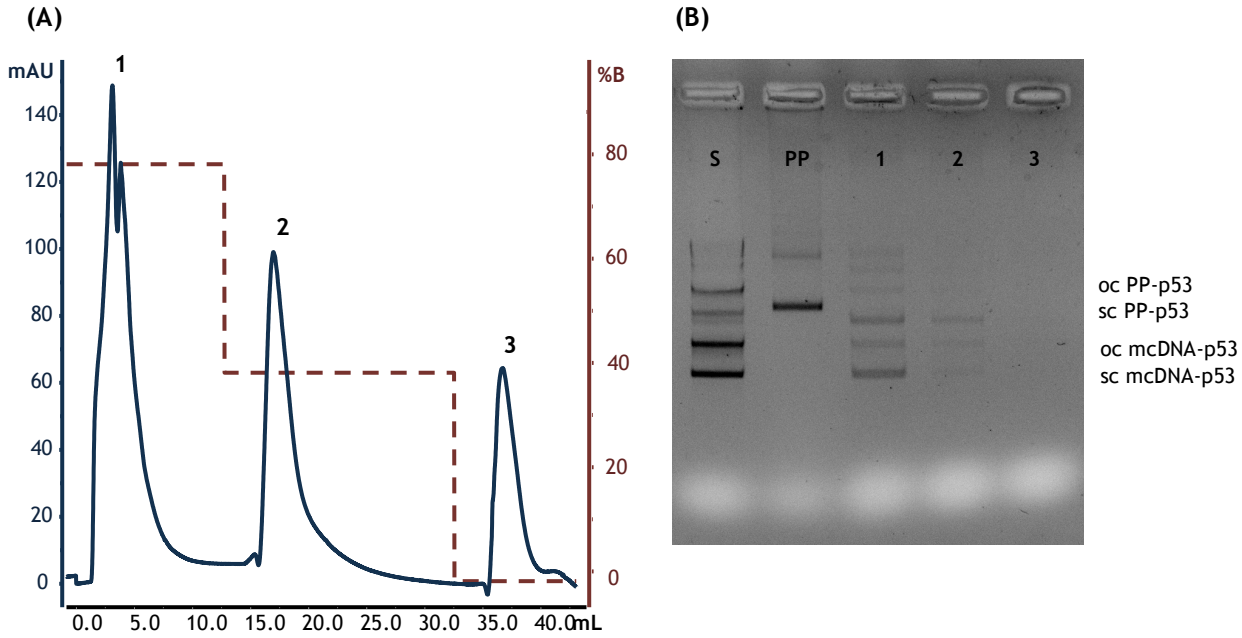


Figure 27. (A) Chromatographic profile of lysate. Elution was performed at 1.0 mL/min by decreasing $(NH_4)_2SO_4$ stepwise gradient from 2.4 M $(NH_4)_2SO_4$ to 1.2 M $(NH_4)_2SO_4$, and finally to 10 mM Tris buffer (pH 8.0), as represented by the % B. **(B)** Agarose gel electrophoresis of samples collected at the column outlet. Fractions corresponding to peaks (1), (2) and (3) are shown in lanes 1-3, respectively. Lane S, mcDNA-p53 sample injected onto the column. Lane PP, PP sample.

The previous assay shows that by decreasing of salt concentration in the binding step to 2.4 M $(NH_4)_2SO_4$, the mixture of mcDNA did not bind to the column and was partially eluted in the flowtrough (peak (1) and lane 1), and some PP was retained, being eluted in peak (2) as it was observed in lane 2 of the agarose electrophoresis. The previous result seems promising and so, it was decided to decrease the salt concentration in order to improve the column selectivity

and to see if the mcDNA would elute completely in the first peak but maintaining the binding of the PP. Therefore, the next chromatographic test was performed by using 2.1 M $(NH_4)_2SO_4$ in the equilibrium buffer at 1 mL/min and after the injection of 200 µL of sample, the first peak was obtained in the flowthgrough. Afterwards, the ionic strength was decreased to 0.9 M $(NH_4)_2SO_4$ and to 10mM Tris buffer at pH 8.0 (Figure 28A). The samples collected at the column outlet were analyzed by 0.8 % agarose gel electrophoresis. (Figure 28B).

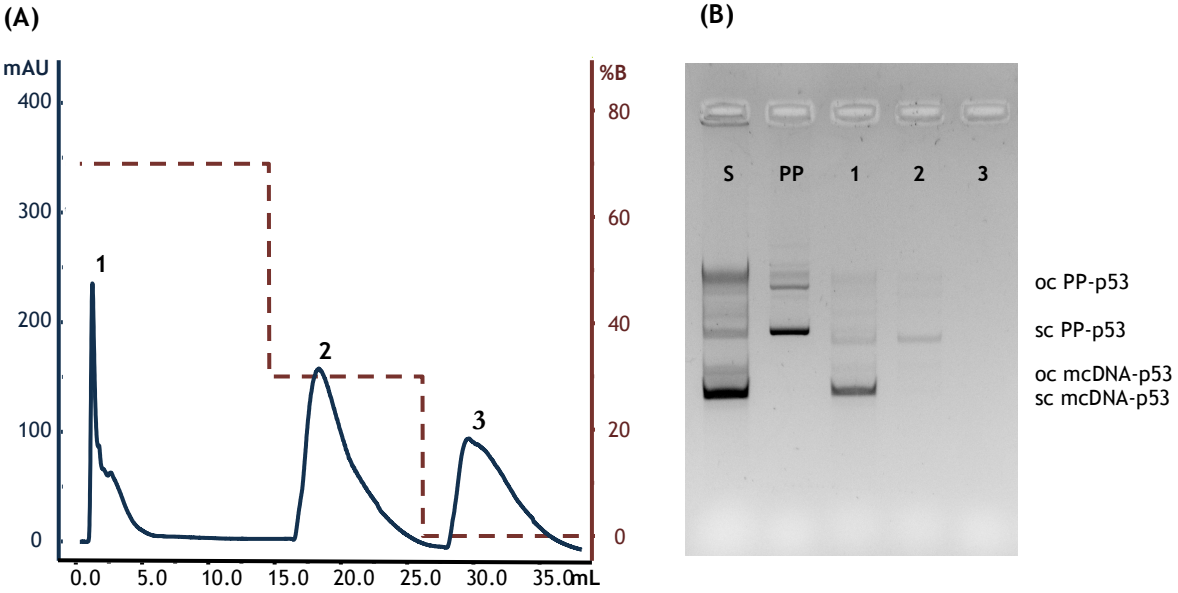


Figure 28. (A) Chromatographic profile of lysate only with decreasing salt concentration. Elution was performed at 1.0 mL/min by decreasing $(NH_4)_2SO_4$ stepwise gradient from 2.1 M $(NH_4)_2SO_4$ to 0.9 M $(NH_4)_2SO_4$ and finally to 10 mM Tris buffer (pH 8.0), as represented by the % B. **(B)** Agarose gel electrophoresis of samples collected at the column outlet. Fractions corresponding to peaks (1), (2) and (3) are shown in lanes 1-3, respectively. Lane S, mcDNA-p53 sample injected onto the column. Lane PP, PP sample.

This chromatographic separation was more satisfactory than previous ones, being achieved the total elution of mcDNA in the first peak with 2.1 M $(NH_4)_2SO_4$, and the retained PP was afterwards eluted with 0.9 M $(NH_4)_2SO_4$. However, some contaminants of higher molecular weight were also eluted together with the mcDNA, revealing that the complete isolation of mcDNA was not accomplished. Another observed problem was the retention of some species in the column that were only eluted during the column regeneration. This regeneration step was conducted by using a flow rate of 0.5 mL/min with 0.1 M NaOH in 30 % isopropanol (v/v) and, finally, Mili-Q water.

Nevertheless, since it is important to obtain the isolated sc mcDNA isoform for future therapeutic applications, it was necessary to try other approaches in order to obtain more satisfactory results and to improve the column selectivity. For that, different salt

concentrations were tested along pH variations. Thus, a chromatographic assay was performed by equilibrating the column with 3 M $(NH_4)_2SO_4$ at pH 6.0 at 1 mL/min and after the injection of 200 µL of sample the binding/elution of the different biomolecules occurred. For the elution step, a linear gradient was applied during 10 min by decreasing the ionic strength and simultaneously increasing the pH from 3 M $(NH_4)_2SO_4$ at pH 6.0 to 2.4 M $(NH_4)_2SO_4$ at pH 8.0. Finally, a stepwise gradient was applied by decreasing only the salt concentration to 0.7M $(NH_4)_2SO_4$ at pH 8.0 and to 10 mM Tris buffer at pH 8.0 (Figure 29A), being eluted one peak in each step. The samples collected at the column outlet were analyzed by 0.8 % agarose gel electrophoresis. (Figure 29B).

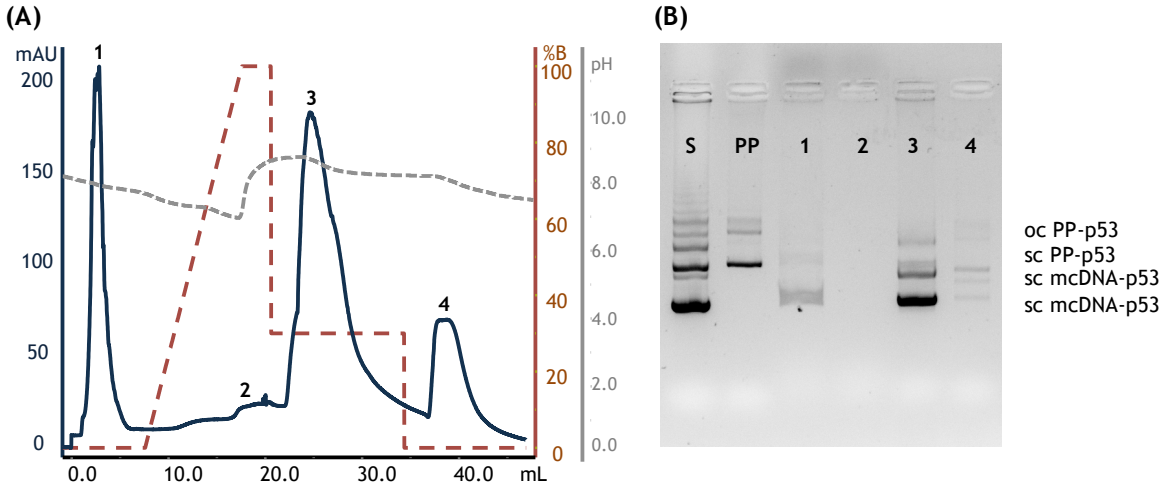


Figure 29. **(A)** Chromatographic profile of lysate with salt and pH variations. Elution was performed at 1.0 mL/min by decreasing $(NH_4)_2SO_4$ linear gradient from 3 M $(NH_4)_2SO_4$ (pH 6.0) to 2.4 M $(NH_4)_2SO_4$ (pH 8.0), followed by decreasing $(NH_4)_2SO_4$ stepwise gradient from 2.4 M $(NH_4)_2SO_4$ (pH 8.0) to 0.7 M $(NH_4)_2SO_4$ (pH 8.0), and finally to 10 mM Tris buffer (pH 8.0), as represented by the % B. **(B)** Agarose gel electrophoresis of samples collected at the column outlet. Fractions corresponding to peaks (1)-(4) are shown in lanes 1-4, respectively. Lane S, mcDNA-p53 sample injected onto the column. Lane PP, PP sample.

With the last strategy, it was possible to obtain the highest yield of sc mcDNA in peak (3), although it still contains some contaminants as it was observed in lane 3 of agarose electrophoresis. This chromatographic profile can be justified by the variation of pH beyond the decrease of ionic strength. Specifically, the acidic pH used in the equilibrium buffer could increase the positive character of arginine and lysine ligands [70], which have basic pKas, favoring the establishment of additional interactions between the sample and the matrix, such as electrostatic interactions, cation-π interactions, hydrogen bonds and hydrogen-π interactions [73]. Thus, it is suggested that the use of pH 6.0 can increase the effective bind of the sc mcDNA to the column, due to the establishment of multiple interactions that are favored with this molecule because of its more compact structure, which increases the charge density,

and the supercoiling phenomenon, which increase the aromatic bases exposition and availability [66]. On the other hand, the increase of pH to 8.0 simultaneously with the decrease of the salt concentration may have weakened the multiple interactions previously referred, allowing a more effective elution of the sc mcDNA as it was observed in this result.

To summarize, from the obtained results we can suggest that the equilibrium strategy with lower concentration of salt seems to favor the selectivity of the column, obtaining a sample of sc mcDNA-p53 with lower content of impurities, although the total recovery of the injected sample has been sacrificed. On the other hand, by using the strategy of effective binding of the sample to the column and manipulating the pH seems to favor the recovery of sc mcDNA-p53 despite containing some impurities. However, the results were not totally satisfactory for future applications, because sc mcDNA was not completely isolated from other contaminants. It is important to improve the purification strategy of this biomolecule since mcDNA technology is recent and has not yet been widely explored and studied, being then a promising alternative to pDNA.

4.4 *In vitro* transfection studies

The principle behind gene therapy relies on successful transfection of cells with nucleic acids, which contain genetic information encoding a particular protein. Therefore, transfection efficiency studies are extremely important in order to preliminary assess the ability of nucleic acids to entre into the cells. For that, after transfection of HeLa cells with PP-p53 and mcDNA-p53 obtained by NZYTech extraction kit, immunocytochemistry, PCR and western blot techniques were performed with the intent of assessing the transfection efficiency, gene transcription and protein expression.

4.4.1 Transfection efficiency analysis

To assess the transfection efficiency of different DNA vectors, the GFP fluorescence was evaluated by immunocytochemistry. The GFP gene is frequently used as an expression reporter and it is present in both DNA vectors (PP-p53 and mcDNA-p53). Thus, it was possible to observe the cells transfected with these vectors because GFP exhibits bright green fluorescence when exposed to UV light. Figure 30 presents different images taken from cultured cells, which were transfected with different DNA vectors.

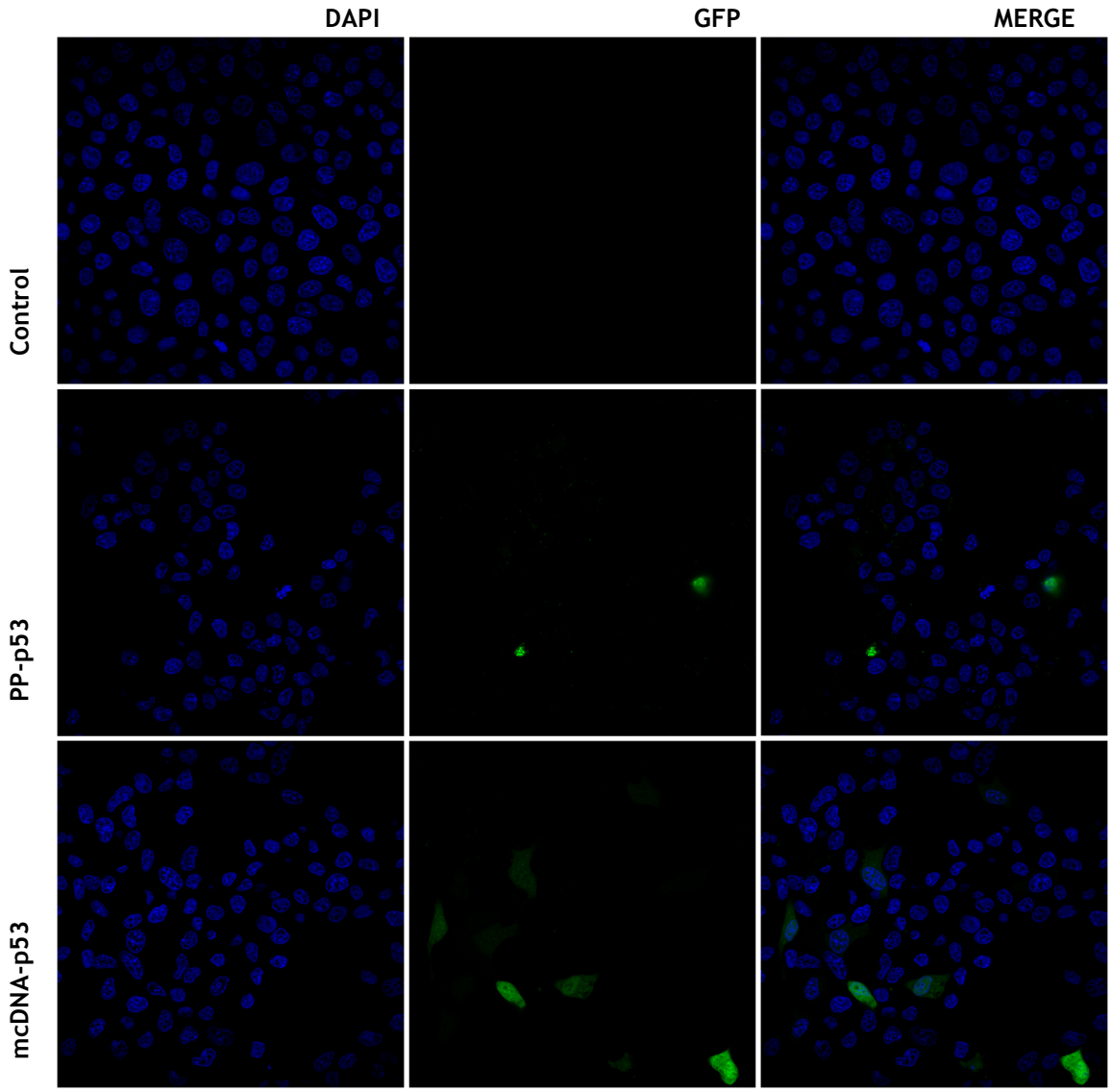


Figure 30. HeLa cells immunocytochemistry images for PP-p53 and mcDNA-p53 staining. The cell nuclei of transfected cells with PP-p53 (48h) and mcDNA-p53 (48h) and non-transfected cells (Control)(48h) were staining with DAPI and transfection efficiency was evaluated through GFP fluorescence.

In Figure 30, it is possible to visualize cell nuclei staining with DAPI. In the cases where there is GFP staining, it is possible to confirm that HeLa cells were transfected with PP-p53 and mcDNA-p53, although a low level of transfection efficiency is notorious. This occurs because 48h of incubation after transfection may not be enough to evaluate the GFP protein expression or because the transfection agent used do not present a high efficiency at inducing cells transfection with DNA vectors. Nevertheless, it can be suggested that mcDNA-p53 had higher transfection efficiency than PP-p53, which is in accordance to the studies and advantages presented for the mcDNA vector. This behavior probably occurs because mcDNA is a smaller

vector than PP vector and devoid of bacterial sequences and antibiotic resistance that allows it to have higher transfection efficiency compared to other vectors [52].

4.4.2 p53 gene transcription

In order to verify the transcription efficiency of p53 gene present into PP and mcDNA vectors, PCR was performed. After 24 and 48 hours of transfection, transfected and non-transfected cells were used for data comparison and the amplification of p53 transcripts was performed with specifically designed primers. After this, samples were analyzed by 1 % agarose gel electrophoresis (Figure 31).

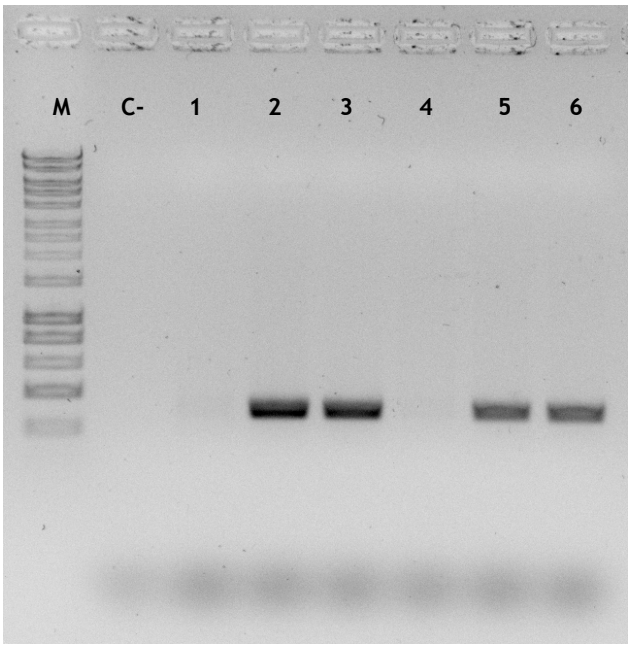


Figure 31. Agarose gel electrophoresis of PCR amplification from HeLa transfected cells study. M- Marker; C- - Negative control of PCR; Lane 1 – Non-transfected cells (24h); Lane 2- Transfected cells with PP-p53 (24h); Lane 3- Transfected cells with mcDNA-p53 (24h); Lane 4 – Non-transfected cells (48h); Lane 5 - Transfected cells with PP-p53 (48h); Lane 6 - Transfected cells with mcDNA-p53 (48h).

As depicted in Figure 31, p53 transcripts were found in transfected cells for 24 h and 48 h. Both electrophoretic bands correspond to the intended amplification molecular weight, 286 bp. These data suggest that the transfection was successfully, occurring the p53 gene transcription with both DNA vectors. It should be noted that cells transfected at 24 h had

higher amplification than at 48 h, probably due to the fact that these cells were already translating some p53-mRNA transcripts into the respective protein or some mRNA was degraded after 48 h since it is an instable molecule. On the other hand, no significant differences were observed between the p53-mRNA transcripts of cells transfected with PP-p53 or with mcDNA-p53 either 24 or 48 hours.

4.4.3 p53 protein expression

After p53 transcripts detection, the correct translation of the transcript into the respective protein was evaluated. Following 72 hours of transfection, transfected and non-transfected cells were submitted at protein extraction to verify p53 protein expression. Making use of western blot technique, a comparison between PP-p53 and mcDNA-p53 transfected cells and non-transfected cells was performed (Figure 33).

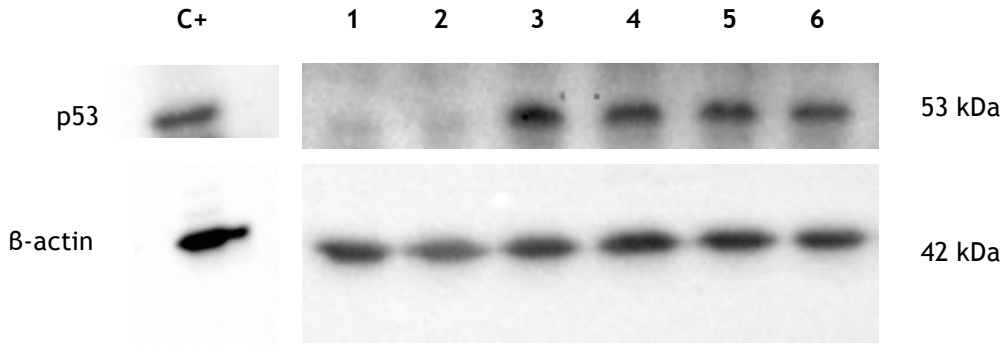


Figure 32. Western Blot of p53 and ß-actin expression. Lane 1, 2 – Non-transfected cells (72h); Lane 3, 4 - Transfected cells with PP-p53 (72h); Lane 5, 6 - Transfected cells with mcDNA-p53 (72h). C+ - Positive control with prostate cancer cell line (DU145).

As shown in Figure 32, it can be observed that the staining p53 protein is identical for cells transfected with both DNA vectors, while no band is presented in the control group (non-transfected cells), as previously suggested by RT-PCR data. These results confirm the expression of p53 protein in cervix cells (HeLa) transfected with PP-p53 and mcDNA-p53, which present low expression or almost absent of the p53 tumor suppressor in the non-transfected cells. It was also used a positive control to p53 expression through DU145 prostate cancer cell line. After the confirmation of the p53 protein presence in transfected cells, normalization with ß-actin housekeeping was performed, by using the same electroblotting membrane. The ß-

actin expression was equal between non-transfected and transfected cells. So, normalization with ß-actin allowed to verify the increased p53 protein expression in transfected cells, compared to non-transfected cells. Likewise, as before it was used a positive control to p53 expression by using DU145 prostate cancer cell line.

To summarize, through transfection studies it was possible to evaluate the transfection efficiency and subsequent gene transcription and translation of PP-p53 and mcDNA-p53 obtained by kit extraction. Firstly, by immunocytochemistry it was observed the PP-p53 and mcDNA-p53 transfection, being more efficient with the mcDNA vector. By RT-PCR it was evaluated the presence of p53 mRNA transcripts into HeLa transfected cells. However, in order to achieve a correct and rigorous comparison between the p53 transcripts resulting from Hela cells transfected with the different used expression vectors, real time PCR should be considered. Finally, p53 protein translation/expression was confirmed by Western blot. Also in this case, additional experiments should be performed in order to increase the replicates and accomplish a significant and statistical evaluation of the produced protein from the cells transfected with PP and mcDNA vectors. In addition, to make a correct comparison of transfection efficiency, p53 transcription and expression between different vectors, it is required the use of the purified sc mcDNA-p53 isoform, since the sample used in previous studies was obtained from a commercial kit and is composed by PP and oc mcDNA molecules beyond the required sc mcDNA.

CHAPTER V

Chapter V – Conclusions and Future Perspectives

Nowadays, millions of people die each year from incurable diseases, because often patients are resistant to drugs or no efficient therapies are available. Particularly, cervical cancer caused by HPV infection kills a lot of women worldwide per year. This situation leads to a great need for new therapeutic strategies, namely based on DNA delivery. Gene therapy has appeared as a potential therapeutic approach, being a good solution to overcome some problems and help to try fighting many diseases, namely several types of cancer. This approach is based on the insertion of genetic material of interest into the cells that can correct a genetic defect or supplement the level of a given protein that is not correctly produced.

However, for pDNA administration, it is necessary to meet the requirements imposed by regulatory agencies, which recommend a homogeneity higher than 97% of sc pDNA, and for that, several chromatographic techniques have been developed in order to achieve a strategy that allows the application of this technology in therapies. This therapeutic biomolecule is obtained by using recombinant organisms. However, these plasmids contain elements necessary for production in bacteria that cause some adverse effects, already described in the literature. In this context, mcDNA arises as useful alternative because it is constituted exclusively by the part of plasmid that contains the eukaryotic expression genes, necessary for therapeutic gene expression.

Thus, the present work was aimed to construct a mcDNA vector encoding p53 to reestablish the levels of this tumor suppressor protein in cervical cancer cells and to induce apoptosis. For that, initially, the mcDNA-p53 construction was started by cloning the p53-encoding sequence into a parental plasmid (PP), and then *E. coli* ZYCY10P3S2T strain was transformed by heat shock with the PP-p53 vector. This strain of *E. coli* was used because it allows the PP recombination in mcDNA and miniplasmid. The mcDNA-p53 production was optimized by variation the conditions of recombination step. A highest mcDNA-p53/PP-p53 ratio was achieved by optimizing the recombination step using 0.01% of L-Arabinose and 2h of induction.

After the production, a new chromatographic column was studied in order to try the sc mcDNA-p53 purification from the other contaminants obtained in the recombination process. For that, it was explored the simultaneous affinity character of two ligands, arginine and lysine, immobilized in the same functional group, triazine. Firstly, ionic and hydrophobic conditions were tested in this matrix, but the best results of purification were obtained by mainly exploiting hydrophobic binding and elution conditions. One of the best obtained results was achieved with equilibrium of the column with 2.1M $(NH_4)_2SO_4$, being eluted a sample in the flowthrough with a good content of mcDNA, and at the same time, part of the PP was retained to the column being eluted with 0.9M $(NH_4)_2SO_4$. However, the complete separation of mcDNA from the remaining contaminants was not accomplished, although it showed some selectivity

between PP and mcDNA. On the other hand, the second best result was carried out by decreasing the ionic strength and simultaneously increasing the pH from pH 6.0 to pH 8.0. This assay allows the elution of a sample with a very good recovery of sc mcDNA-p53, although the sample still presents some contaminants.

Afterwards, *in vitro* transfection studies were conducted by using HeLA cells, in order to evaluate the transfection efficiency of PP and mcDNA vectors and the p53 expression. Firstly, immunocytochemistry was performed and it was concluded that the transfection with PP and mcDNA has occurred, being more efficient with mcDNA. Then, RT-PCR was performed to evaluate the presence of p53 mRNA transcripts in transfected cells. Finally, p53 protein expression was confirmed by Western blot, also in transfected cells.

In a future perspective, it is necessary to optimize the purification process of this biomolecule, requiring a further characterization of this new chromatographic column through the study of interactions established between the sample and this new chromatographic matrix by using the Surface Plasmon Resonance and Nuclear Magnetic Resonance. These studies can allow to explore new conditions in order to guarantee a better selectivity for the mcDNA. It is also necessary to evaluate the purity of the purified sample by determination of proteins by the Bicinchoninic Acid assay (BCA), endotoxins by Limulus Amebocyte Lysate assay (LAL), and gDNA by real time PCR. Finally, it will be important to implement an analytical method to evaluate the percentage of the sc isoform of mcDNA and the recovery obtained in the purification strategy.

Since mcDNA-p53 is a vector with a therapeutic gene for future application, more *in vitro* transfection studies in cervical cancer cells are required by using the purified sc mcDNA-p53 isoform to evaluate the differences of transfection efficiency between this biomolecule and PP. Furthermore, real time PCR and more western blot experiments were necessary to carry out in order to be possible a realistic and statistical evaluation and comparison of the p53 transcription and expression in function of different vectors. Finally, flow cytometry studies may also be performed to evaluate the biological effect of the p53 level conferred by the mcDNA vector on cervical cancer cells, namely the apoptosis induction.

To conclude, with this work it was possible to construct a mcDNA vector with a therapeutic gene, optimizing its production, revealing potential to be developed a gene therapy in the future, and thus treat cervical cancer.

CHAPTER VI

Chapter VI – Bibliography

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